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A CHEMICAL STUDY OF THE SPECIFIC ELEMENTS OF TUBERCULIN. I.

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(Received for publication, September 21, 1925.)

The work to be described in this paper was undertaken with the object of clearing up the conflicting reports in the literature as to the protein or non-protein nature of the substance in tuberculin to which is due its specifically toxic reaction in the body of the tuberculous animal. The literature on the subject has been summarized by Long (1), and need not here be reviewed in detail. Certain of the more recent studies will be considered in relation to the present paper in the discussion.

It may be stated at the outset that we have not been able to arrive at a satisfactory conclusion as to the chemical nature of the fraction in question, but certain points in connection with the chemistry of the complex material known as old tuberculin have been very clearly demonstrated. Some of these we have already reported in a preliminary way (Mueller (2), Zinsser and Mueller (3)). In view of the report by Long and Seibert (4) which has just appeared, and the recent work of Laidlaw and Dudley (5), it seems justifiable to present the work at this stage. Possibly the complete account of Long and Seibert's work, which is stated to be in press, will make it unnecessary to continue further in our efforts to purify the toxic material. The work of Laidlaw and Dudley will be discussed in the second paper (6) on this general subject.

The work of Zinsser and Zinsser and Parker (7) on residue antigens of the tubercle bacillus and other microorganisms furnished the point of departure for the experiments which follow.

It was shown by these writers that a slightly alkaline extract of tubercle bacilli freed as far as possible from protein by acid precipitation and heat coagulation,

contained a substance, precipitable by alcohol, which produced typical skin reactions in tuberculous guinea pigs. A specific precipitin reaction was also given by this material with the serum of an animal (sheep or rabbit) injected repeatedly with dead tubercle bacilli. Similar extracts prepared from meningococci, pneumococci, and influenza bacilli also precipitated specifically with their homologous immune sera. It was shown, too, that the substance separated by slight acidification of the alkaline tubercle extracts (nucleoprotein?) gave, like the partially purified filtrate, a strongly positive skin test in tuberculous guinea pigs, the reason for which could not be satisfactorily explained at that time.

That these "residue antigens" from many types of bacteria and related organisms contain non-protein substances of the nature of polysaccharides, which appear to be responsible in large measure for the specific precipitating, agglutinating, and complement-fixing properties of bacteria, is a fact that has been abundantly confirmed and amplified (8). It was the writer's purpose to prepare this material from tubercle bacilli, study its properties and determine whether in the case of this organism, too, a nitrogen-free and therefore non-protein substance was produced, to which were attributable the specific properties of tuberculin including its toxic action in tuberculous animals.

Because old tuberculin is highly potent both in animal tests and *in vitro* immune reactions, and is free from the more or less highly insoluble bacterial bodies, it was chosen rather than the bacilli themselves as the material to be used in attempted purification.

Toxic action was determined by the intracutaneous injection of about 0.1 cc. of a 1:10 dilution of the O.T., or an equivalent amount of the various fractions, into guinea pigs which had been inoculated at least 3 weeks previously with a suspension of living tubercle bacilli. Strain H 37 from Saranac has been used in all the work to be reported. The reactions were, of course, controlled with similar injections into normal animals. The precipitin reaction was carried out by the ring method, using a few drops of undiluted serum from a rabbit treated repeatedly at 4 to 5 day intervals with a saline suspension of heat-killed tubercle bacilli, and layering over it in narrow tubes dilutions in saline of the tuberculin or its fractions.

The earlier unsuccessful attempts at purification need not be recounted. Preparations were tested sometimes by the simple and rapid precipitin reaction, at other times by the skin test, until it became apparent that discrepancies in results were obtained by the

two methods. We then tested each preparation or fraction in both ways and were soon forced to the conclusion that the substance giving the precipitin test was quite separate and distinct from that producing a skin reaction. This may be illustrated in three different ways, none of which seemed applicable to large scale development. Protocols will be omitted in the interest of brevity.

Proteolytic Enzymes.

A solution of old tuberculin treated under appropriate conditions with pepsin or trypsin for some time, gradually loses its power to give a skin test, which power finally disappears completely, leaving the precipitating property with immune serum unimpaired.

Acetic Acid Precipitation.

If a quantity of O.T. prepared in the usual way from a 6 weeks glycerol-broth culture of H 37 be precipitated with 10 volumes of alcohol, and the precipitate redissolved and again precipitated once or twice in the same way, a preparation is obtained giving both types of reaction. If, to an aqueous solution of this material, enough acetic acid is added to produce maximum precipitation, the precipitate will give only the skin test, while the filtrate will still give both.

Tannic Acid Precipitation.

If some of the alcohol precipitate of O.T. or some of the acetic acid filtrate, prepared as above, is precipitated with tannic acid, filtered, and the filtrate freed from the excess of tannic acid, it will give a strong precipitin test, but no skin reaction whatever.

The presence of considerable quantities of foreign organic material in O.T. made from meat broth proved to be exceedingly troublesome, and for the preparation of larger quantities of material from which we hoped to separate both active substances a "synthetic" broth of the following formula, for which we are indebted to Petroff, was used:

KH_2PO_4	5.0 gm.
$MgSO_4$	0.6 gm.
Magnesium citrate	2.6 gm.
Asparagine	5.0 gm.
Glycerol	20 cc.
Water	1000 cc., adjusted to pH 7.0 with NaOH.

Upon this medium in Kolle flasks, growth of Strain H 37 was practically as rapid and luxurious as upon meat broth, and the properties of the resulting O.T. were not markedly inferior. Using tuberculin prepared in this way, separation of the toxic factor and the precipitating factor proved exceedingly simple.

6 week cultures of the tubercle bacillus are sterilized in an Arnold sterilizer for 1 hour and evaporated in large porcelain dishes on the water bath to about one-tenth the original volume. The bacilli are either filtered out at this point or strained off through cheese-cloth before evaporation. It does not seem to be particularly important whether evaporation takes place in the presence or absence of the growth. The resulting syrupy liquid is precipitated by adding slowly, and with stirring, 2 volumes of 96 per cent alcohol. The precipitate is either allowed to settle overnight, and the supernatant decanted, or else is centrifuged down; it contains very little of either active fraction and is discarded. To the supernatant is added enough 96 per cent alcohol to make a total of 5 volumes of alcohol. This precipitate is separated as in the case of the first one, and is found to contain practically all the precipitating fraction and to give almost no skin reaction. It usually consists of two layers, a lower liquid layer and an upper semi-solid layer. Considerable of the material in both layers is inorganic, and the residue antigen seems to divide itself between both, so that neither can be discarded. The purification of this fraction will be the subject of a separate paper.

The 5 volume alcohol filtrate, when freed from alcohol by evaporation, and suitably diluted, gives a strong specific skin reaction and almost no precipitin test. The toxic material can be precipitated by first concentrating to about the volume of the original O.T., or somewhat less, on the water bath, and then adding 5 volumes of acetone. This fraction also contains considerable inorganic material and gives a strong biuret reaction. It was freed from a large part of the impurities by dialysis in fish bladders against running water, and it was found that part, but not all, of the toxic material could be separated from the dialyzed solution by acidification with dilute acetic acid, and, also, that practically all the active material could be precipitated by saturating with ammonium sulfate or sodium sulfate at 34°. It contained a considerable amount of protein material, but was not by any means a pure protein as shown by the nitrogen determination. Moreover, while it gave skin reactions at this stage, it was necessary to use a considerably larger quantity for strong reactions than should have been the case. We have been unable to assure ourselves as to whether this indicates a gradual loss of an active component, or a specific alteration in properties of the product now on hand through heating, exposure to alcohol and acetone, oxidation, or some other factor.

Details of Preparation.

6 liters of O.T., prepared from the synthetic media described above, were precipitated first with 2 and then with 5 volumes of alcohol. The 2 volume precipitate was discarded, the 2 to 5 volume precipitate used as the source of residue antigen to be described in the following paper. The 5 volume filtrate was concentrated on the water bath to about 2000 cc. and precipitated with 5 volumes of acetone. After standing overnight, the clear acetone supernatant was removed and discarded. The precipitate was dissolved in about 500 cc. of water, warmed on the steam bath for a short time to remove a part of the remaining acetone, and dialyzed in several fish bladders against running water for 18 hours. The dialyzed solutions were again combined, concentrated on the water bath in front of a fan to about 500 cc., some insoluble material centrifuged out, and the clear, brown solution precipitated with a mixture of 1000 cc. alcohol and 2500 cc. acetone. After standing overnight, the practically clear supernatant was removed and discarded, although it still contained a small amount of the active substance, as shown by a skin reaction. The precipitate was dried by stirring with acetone and then with ether, finally transferring to a vacuum desiccator. The yield was 16.4 gm., which contained 7.7 per cent nitrogen. 0.1 mg. of this material produces a good skin reaction in tuberculous guinea pigs.

Combining with the above product enough similarly prepared material to make a total of 22 gm., the entire quantity was dissolved in 400 cc. water, and a few cc. of $\frac{N}{1}$ NOOH. After centrifuging from a small quantity of insoluble material, the solution was saturated with Na_2SO_4 at 34°, and the precipitate filtered off. The filtrate was practically inactive and was discarded. The precipitate was dissolved in 300 cc. water. The addition of a few drops of $\frac{N}{1}$ NOOH causes the slightly turbid solution to become practically clear. About 3 cc. of glacial acetic acid were added until maximum precipitation took place. The pH was then about 3.4. The precipitate was centrifuged down, redissolved in 100 cc. water, and again precipitated with acetic acid. The precipitate, after removal by centrifugation was dried *in vacuo* after suspending in acetone and in ether. The combined supernatant fluids from the two acetic acid precipitations were precipitated by several volumes of acetone, and this precipitate also dried and preserved.

The yields were respectively 2.0 gm. from the acetic acid precipitate and 1.5 gm. from the supernatant. The former contains 3.3 per cent N, and the latter 5.5 per cent by the colorimetric micro Kjeldahl method. Both fractions give a weak precipitin reaction at a dilution of 1:100, and the acetic precipitate fraction a doubtful reaction at 1:1000 in 2 hours. Skin reactions on tuberculous guinea pigs are positive with both fractions when 0.1 cc. of a 1–10,000 solution is used, the precipitate

fraction being stronger, and corresponding roughly to the reaction in the same pigs given by a 1:100 dilution of O.T. Both fractions give well marked biuret and Millon tests for protein in 1 per cent solution and an exceedingly faint Molisch test for carbohydrate in a 0.2 per cent solution.

DISCUSSION.

We believe it has not been appreciated by those working with tuberculin that the test-tube reactions of precipitation and complement fixation might be due to an essentially different component of the material from that producing its specific physiological effect. This separation has already been reported by us in preliminary communications (1, 2) and of course follows also from the work of Laidlaw and Dudley. It is therefore apparent that the various methods which have been suggested for the standardization of tuberculin for therapeutic or diagnostic use, based upon the precipitin or complement fixation reaction (9) are erroneously conceived and therefore of little value. We have not carried out complement-fixing tests with the skin-toxic fractions of tuberculin but fixation is readily obtained by solutions of the carbohydrate residue antigen of the tubercle bacillus, as one should expect from the now generally accepted relationship between fixation and precipitation. These reactions were carried out by Dr. S. Litarczek in this laboratory, to whom we wish to take this opportunity of expressing our thanks. We are unable to explain the results reported by Dienes, Schoenheit, and Scheff (10) who were unable to obtain evidence of complement fixation with fractions of the tubercle bacilli other than protein.

As already stated, it is quite impossible to say whether the final product obtained in the form of an acetic acid precipitate represents even an approximately pure tuberculin active material or not. It obviously contains protein, but only to the extent of perhaps 20 to 25 per cent. The remaining material may be an impurity or it may be that the protein is combined with a non-nitrogenous material in some form. If skin reactions of approximately the same intensity are given by a 1:100 dilution of O.T. and a 1–10,000 solution of the partially purified product, it can be calculated that about 50 times as much of the latter is required as would be expected if the substance had

been isolated without loss and without alteration in properties. In other words, 0.01 mg. is required, while one should expect to obtain a reaction with 0.0002 mg., if 2 gm. represent all of the active material actually present in 10 liters of O.T. Of course large losses have occurred at each stage of purification. It is almost impossible by the methods available to approach quantitative judgment in this problem. We are inclined to the view, however, that in addition to actual losses, there has probably been some alteration in the specificity of our product. Until it can be determined in what proportion loss and alteration enter into the results, it is of no great moment to study in detail the chemistry of our product. It is likewise not easy to compare the activity of our preparation with that obtained by Long and Seibert. These investigators state that their preparation is a non-coagulable, water-soluble protein which gives a strongly positive reaction in the testis of a tuberculous guinea pig in a quantity of 0.0001 mg.; that is, 1 per cent of the amount of our material required for a definitely positive necrotic skin reaction. The information as to the relative quantities of O.T. which give positive skin reactions and intratesticular reactions does not seem to be available. Presumably much less would be required for the latter type of reaction.

CONCLUSIONS.

1. The specific precipitin reaction and the skin reaction given by old tuberculin are attributable to two separate substances present in this material. The cause of the reaction first mentioned is a non-protein gum which will be fully described in the following paper. The cause of the skin reaction is probably but not surely a protein.

2. Methods which have been suggested for the standardization of tuberculin by precipitin or complement fixation reactions should be revised.

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A CHEMICAL STUDY OF THE SPECIFIC ELEMENTS OF TUBERCULIN.

II. The Preparation of Residue Antigen from Old Tuberculin.

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(Received for publication, September 21, 1925.)

Substances for which the term "residue antigen" has been suggested by Zinsser (1) and "specific soluble substance" by Heidelberger and Avery (2) have been prepared by the latter workers (3) from the three fixed types of Pneumococcus, by Lancefield (4) from *Streptococcus viridans* and by ourselves (5) from a strain of yeast and one of Friedländer's bacillus. We have also reported in preliminary communications (6) the preparation of a similar material from tuberculin. The present paper contains an account of the method of preparation and purification, and a partial study of the properties of this material. A paper by Laidlaw and Dudley (7) which has just appeared describes what is obviously the same substance obtained from tubercle bacilli themselves, while our preparation is from broth filtrates of cultures.

Method of Preparation.

About 10 liters of O.T., prepared by evaporating to one-tenth the synthetic media[1] upon which Strain H 37 of Saranac had grown for 6 weeks, was precipitated by the addition of 2 volumes of alcohol. After standing overnight the supernatant was drawn off, 3 more volumes of alcohol added, and again allowed to settle overnight. This precipitate, after removal of the alcohol, was dialyzed for 48 hours, in several fish bladders, the dialyzed solution concentrated somewhat on the water bath, and precipitated with 10 volumes of alcohol, after adding 1 or 2 gm. of sodium acetate. The precipitate was dried in the desiccator, the yield being 18 gm.

About one-third of this was used in various attempts to remove the 3 to 4 per

[1] Formula given in preceding paper.

cent nitrogen it contained before a method was worked out which seemed to be satisfactory. It is not clear in what form the nitrogen is present, since it is not readily removed by reagents which would be expected to precipitate either proteins or nucleic acids.

The remaining 12 gm. were dissolved in 200 cc. hot water, centrifuged from some insoluble material, and the supernatant acidified with acetic acid. Some precipitate separates and, after centrifuging, the supernatant was made alkaline with NaOH to about an $\frac{N}{4}$ concentration and another rather bulky precipitate centrifuged down. These precipitates were all discarded. The alkaline supernatant was precipitated with 9 volumes of 96 per cent alcohol. It was centrifuged and formed a yellowish brown, gummy layer. This was dissolved in 100 cc. water and neutralized with acetic acid. Copper sulfate was added which caused a moderate amount of a flocculent precipitate to separate, which was discarded. The solution was then acidified with HCl and precipitated with 10 volumes alcohol, and centrifuged. The precipitate was a dark green gum. This was redissolved in 100 cc. water, again acidified with HCl, and precipitated with alcohol as before. Copper was still present, and the material, dissolved in a little water, was dialyzed 2 days with occasional addition of a few drops of dilute HCl. Practically all the copper had now disappeared, and the solution was yellowish brown, showing some fluorescence. It was concentrated to about 50 cc. on the water bath and after cooling, 5 cc. antiformin added. This changed the color at once to a deep wine-red with marked fluorescence. After standing 2 hours at room temperature, 2 to 3 cc. more antiformin were added and 10 minutes later it was precipitated by 10 volumes alcohol. The centrifuged gum was redissolved in 50 cc. water, about 0.2 gm. NaOH added, and again precipitated by alcohol. The precipitate was dissolved in 50 cc. water, slightly acidified with HCl, changing the red color to light greenish yellow, and 8 volumes alcohol added. The precipitate was once more dissolved and precipitated, this time from neutral solution. The final precipitate was a perfectly transparent, greenish yellow gum which had at no stage shown any tendency to separate in a flocculent form. It was therefore rinsed with strong alcohol, transferred to a vacuum desiccator over $CaCl_2$, and strongly evacuated for 2 days, when it dried to a rather spongy mass of brittle, transparent, slightly greenish material which could readily be scraped out of the centrifuge tubes and powdered. The yield was about 3.5 gm. A ring precipitin test is given by this substance up to a dilution of 1:2,000,000. The skin test is negative at 1:1000.

This material contains 0.3 per cent nitrogen and a very slight trace of phosphorus. 3 per cent ash containing a trace of copper is also present. It does not reduce Benedict's solution, but gives a very strong alpha-naphthol test for carbohydrate. It gives no precipitate with picric, tannic, or phosphotungstic acid, nor with lead acetate, neutral or basic, copper sulfate, uranium nitrate, silver nitrate, mercuric nitrate, nor with safranine. In fact we have found nothing which seems to be suitable for its precipitation.

The optical rotation was determined on a 3 per cent aqueous solution in a 1 cm.

tube. $[\alpha]_D = +17.3°$. After adding sufficient concentrated HCl to make about an $\frac{N}{2}$ solution, $[\alpha]_D = +24.2°$. The solution was boiled under a reflux for 2 hours at which time the specific rotation had dropped to $-22.9°$. At the end of 3 hours no further change had taken place, but heating was continued for a total of 4 hours. By this time a brownish red color had developed which made further optical measurements impossible. A part of this solution was then used for a quantitative determination of reducing substances. Using Benedict's solution, reduction was obtained corresponding to 99 per cent glucose. Both the original gum and the solution of reducing substances give a strong green color with orcinol, evidence of the presence of either a pentose or glucuronic acid. It has not been possible to prepare an osazone from the hydrolyzed products of the gum in a form which could be identified. Nothing separates during an hour's heating on the water bath except a little brown tar. Upon cooling a yellow, apparently semicrystalline material forms, which either melts or goes back into solution very readily upon warming. By treating some of the hydrolyzed solution, after partially neutralizing the HCl, with a solution of *p*-bromophenylhydrazine hydrochloride and sodium acetate, and heating at 60° on the water bath for an hour, nothing separates except a small quantity of brown oily material. It would therefore appear that glucuronic acid is not present in any quantity.

By the distillation of the gum with 3.3 N HCl by the standard method for determining pentoses, 0.1497 gm. gave sufficient furfural to yield 0.0763 gm. of the phloroglucinol derivative. This calculates to 54.8 per cent of the material as pentose, or 48.3 per cent as a pentosan. The filtrate from the dark colored phloroglucinol precipitate is light greenish yellow, and shows no trace of red, and on standing several days no red precipitate separated, which was interpreted as showing the absence of a methylpentose.

Obviously this gum corresponds very closely to that described by Laidlaw and Dudley. Their yield of material, prepared from whole tubercle bacilli, would appear to be somewhat lower than ours from a corresponding quantity of broth filtrate. The possibilities for loss in their method would appear also to be somewhat greater than in our own. It is extremely interesting to learn that these residue substances may be split off of the protein-gum complex in which they apparently exist in the whole bacteria by relatively drastic treatment with hot alkali, without alteration in their specific properties. The resistance of these substances to alkali, even when hot, to antiformin and to cold acids is perhaps one of the most striking features in connection with all the work which has appeared.

We have not attempted to immunize rabbits with our product,

assuming that, as in the case of other similar materials with which this has been tried, it would be without effect. Laidlaw and Dudley state that such is the case with their product, since it neither induces antibody formation nor stimulates it when immunization is commenced with whole tubercle bacilli.

As stated in the preceding paper, the gum fixes complement in the presence of immune serum and since it does not give a positive skin reaction in tuberculous guinea pigs, it is obvious that tuberculin for diagnostic or therapeutic use cannot be standardized by either the precipitin (8) or complement fixation (9) test.

SUMMARY.

A non-protein gum has been isolated from broth filtrates of human tubercle bacilli grown on a simple medium. It fixes complement and precipitates in high dilution in the presence of homologous immune serum, but fails to give a skin test in tuberculous animals. The preparation and some of the chemical properties of the substance are described.

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A STUDY OF HEMOLYTIC STREPTOCOCCI IN ACUTE RHEUMATIC FEVER, WITH AN ANALYSIS OF THE ANTIGENIC RELATIONSHIPS EXISTING AMONG CERTAIN STRAINS.

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INTRODUCTION.

The part played by streptococci in acute rheumatic fever has long been a subject for discussion, with the chief attention directed towards *Streptococcus viridans*. Hemolytic strains have, however, been isolated at times from the blood and other sources in patients with this disease. It has long been noticed that many patients give a history of a sore throat shortly before the onset of their acute arthritis. Of 122 admissions for rheumatic fever to the Hospital of The Rockefeller Institute during the last few years, a history of a recent acute sore throat was obtained from 62 patients—or, roughly, 50 per cent. Since one of the commonest organisms associated with an acute tonsillitis or pharyngitis is a hemolytic streptococcus, it seemed of interest to seek for, and, if found, to study these organisms in the throats of patients early in the course of rheumatic fever. A difficulty here arose: by the time the patients with arthritis enter the hospital most of them have more or less completely recovered from their throat infection and show at most slight redness of the tonsils or pharynx. During the past 5 years at this Hospital, only 5 rheumatic fever patients have been seen in whom the throat infection was really active at the time of admission. Throat cultures were made from 4 patients within 10 days of the onset of their acute throat infection: in two, hemolytic streptococci were the predominating organisms; in the third, there were only a few hemolytic streptococcus colonies; in the fourth, there was an almost pure culture of *Streptococcus viridans*.

It has been shown that in several diseases associated with hemolytic streptococci these have special serological characters. Tunnicliff (1), Dochez and Bliss (2), Gordon (3), and others found that streptococci from patients with scarlet fever fall into a definite serological group. Tunnicliff (4) and Birkhaug (5) have demonstrated a similar relationship among erysipelas strains. Dochez, Avery, and Lancefield (6), also, found 4 well defined groups in a pneumonia epidemic in Texas. We accordingly endeavoured to ascertain if the strains isolated from patients with rheumatic fever were serologically related. The importance of isolating such strains as early as possible in the disease is evident especially since Eagles (7) has presented evidence to show that the streptococci from the fauces of patients with scarlet fever may, during the early weeks of the disease, change in their power to be agglutinated by a single immune serum. No attempt will be made in this paper to discuss what part, if any, is played by hemolytic streptococci in the etiology of rheumatic fever.

Sources of Material.

13 strains of hemolytic streptococci were isolated from patients with rheumatic fever, in most instances by means of throat swabs; and compared with them were 14 strains from other sources; these included the 4 type strains of Dochez, Avery, and Lancefield (6), (S3, S23, S60, and S84), Gordon's Type I *Streptococcus pyogenes* strain, a scarlet fever strain from Dochez (NY5), and an erysipelas strain from Dr. Harold L. Amoss (B3); the others were mostly from infections of the respiratory tract. These 27 strains were studied from the standpoint of sugar fermentation, agglutination, and agglutinin absorption. For the serological studies sera were prepared against 4 "rheumatic fever" and 5 other strains.

Table I shows the source of the strains and some of their cultural characteristics. Their ability to ferment carbohydrates was tested by growth in Hiss serum water with Andrade's indicator; the tubes were incubated for at least 4 days before being read; growth was verified by subcultures on blood agar plates. Special mention must be made of 3 of the "rheumatic fever" strains against which sera were prepared. T10 is of particular interest: it was recovered from an old rheumatic fever patient, A. M., a girl of 14, who was admitted

on account of a slight bronchitis. While under observation she developed acute angina in the course of an epidemic of sore throat which was present in the ward; from her throat nearly a pure culture of hemolytic streptococci (T10) was obtained. 4 weeks later she developed mild acute polyarthritis. 6 weeks after the onset of angina, large numbers of hemolytic streptococci (T30) were still present in her throat and, unlike most of the streptococci which Eagles studied from patients with scarlet fever, these seemed to be serologically identical with those recovered earlier. From another patient, A. B., a girl aged 16, dying of acute rheumatic carditis on the 16th day of her disease, streptococci (T20 and T25), apparently serologically identical, were recovered from the pericardium and tonsil respectively. T27b was a remarkable organism recovered from a subcutaneous fibroid nodule excised at biopsy from a boy aged 7. As shown in Table I, its sugar fermentations were unusual; it hemolysed blood on plates, but would never do so in liquid media; a final pH of 4.5 was attained in dextrose blood broth, as would be expected with a bovine strain. In addition to this strain only 4 of those studied failed to fall by their sugar fermentations into the classical *Streptococcus pyogenes* group.

Technique.

Sera were prepared by immunizing rabbits intravenously. The animals were given injections on 4 successive days, then 4 days rest, and so on; 3 courses were usually sufficient. For the first 6 injections the rabbits received heat-killed cultures which were increased gradually from 5 to 15 cc.; subsequently live cultures, starting with 0.5 cc. and increasing to 5 cc., were injected. 5 days after the last inoculation a test bleeding was made; if this was satisfactory the animal was exsanguinated and the serum stored on ice without preservatives. The sera all agglutinated the homologous organism to a dilution of 1:1280, and frequently to 1:2560.

The agglutination technique was similar to that described by Dochez, Avery, and Lancefield (6): buffered broth at a pH of 7.6 was used for the cultures and for making all dilutions; the sedimented organisms were washed once and resuspended in similar broth. Thus suspensions sufficiently stable for agglutination tests were obtained with all but 5 of the 27 strains. Unless the suspension was quite stable in the control tubes with broth and normal rabbit serum the experiment was disregarded. Readings were made after 1½ hours (in earlier experiments 2 hours) in the incubator at 56°C.

TABLE I.

Source of Streptococci and Action on Carbohydrates.

Strain.	Patient.	Disease.	Organ.	Abundance.	Length of time between onset of sore throat and time of culture.	Lactose.	Saccharose.	Raffinose.	Inulin.	Salicin.	Mannitol.	Litmus milk.
					days							
T10	A.M.	Rheumatic fever.	Throat.	Nearly pure.	1	+	+	0	0	+	+	AC
T20	A.B.	" "	Pericardium (autopsy).	Scanty. Mixed with green streptococci.		+	+	0	0	+	0	AC
T25	A.B.	" "	Tonsil (autopsy).	Predominant organism.	32	+	+	0	0	+	0	AC
T27b	B.V.	" "	Subcutaneous nodule.	Scanty. Green streptococci also recovered.		(±)	+	+	+	+	0	0
T30	A.M.	" "	Throat.	50 per cent of colonies hemolytic.	42	+	+	0	0	+	+	AC
T40	A.McC.	" "	"	About 8 hemolytic colonies on plate.	60	+	+	0	0	+	0	AC
T43	M.K.	" "	Heart's blood (autopsy).	Pure culture.		+	+	0	0	+	0	AC
T61	S.R.	" "	Throat.		48	+	+	0	0	+	0	AC
T77	J.S.	" "	"	Few colonies.	25	+	+	0	0	+	0	AC
T81	M.S.	" "	"	Only 3 hemolytic colonies on a plate.	16	+	+	0	0	+	0	AC
T88	A.T.	" "	"	Fair number of colonies.	?	0	+	0	0	+	0	0
T102	M.S.	" "	Tonsil.	Nearly pure cultures.	63	+	+	0	0	+	0	AC
T105	E.R.	" "	Throat.	Very many colonies.	292	+	+	0	0	+	0	AC

S3	Texas patient 1918	Bronchopneumonia.	Lung (autopsy).			+	+	0	0	+	0	AC
S23	" "	Lobar pneumonia.	Throat.		?	+	+	0	0	+	0	AC
S60	" "	Measles.	"		?	+	+	0	0	+	+	A
S84	" "	Bronchopneumonia.	Pleural fluid.			+	+	0	0	+	0	A
Gordon Type I	London patient.	Abortion.	?			+	+	0	0	+	0	A
T58	P.A.	Lobar pneumonia.	Lung (autopsy).			+	+	0	0	+	0	AC
T70	G.M.	Sore throat.	Throat.	Nearly pure culture.	1	+	+	0	0	+	0	AC
T79	A.R.	Pneumonia.	Lung (autopsy).			+	+	0	0	+	0	AC
T92	C.N.	Sore throat.	Throat.	Many colonies.	7	+	+	0	0	+	0	AC
T95	E.E.	Septicemia.	Blood.	Pure culture.		+	+	0	0	+	0	AC
T99	F.Q.	Pneumonia.	"	" "		+	+	0	0	+	0	AC
T107	R.T.	Sore throat.	Throat.	Many colonies.	3	+	+	0	0	+	0	AC
NY5	?	Scarlet fever.	"		?	+	+	0	0	+	0	A
Erysipelas B3	H.A.	Erysipelas.	Blood.			+	+	0	0	+	0	AC

+ indicates fermentation with acid formation.

In the litmus milk column, A indicates acid formation and C clotting.

Agglutinin absorption was used as a check on the agglutination in all cases; by its aid we were able to study even the 5 strains in which the growth was persistently granular. Serum with a titre of at least 1:1280 was diluted 40 times with buffered broth; 0.75 cc. portions (in earlier experiment 1 cc.) of this diluted serum were each absorbed once with the centrifuged deposit from 50 cc. of heavy 18 hour growths of the various strains; these proportions were found sufficient to absorb the agglutinins from a serum by its homologous strain. After absorbing for 2 hours in a water bath at 37°C. the mixtures of serum and streptococci were centrifuged; the serum was pipetted off and set up against its homologous organism in dilutions of 1:80, 1:320, and 1:1280. Control tubes were always set up containing respectively a streptococcal suspension with broth, with normal rabbit serum, with unabsorbed immune serum, and with serum absorbed by the homologous organism. As it was found that live or heat-killed organisms were equally utilizable, living cultures were employed throughout. A typical protocol is shown in Table II.

TABLE II.

Protocol of an Agglutinin Absorption Experiment with S23 Serum.

All sera, both absorbed and unabsorbed, tested against Strain S23.

Serum dilution.	Absorbed by Strain S23.	Absorbed by Strain S60.	Absorbed by Strain S84.	Absorbed by Strain T20.	Absorbed by Strain T58.	Absorbed by Strain T61.	Absorbed by Strain T70.	Absorbed by Strain T77.	Absorbed by Strain T81.	Absorbed by Strain T88.	Absorbed by Strain T92.	Absorbed by Strain T95.	Absorbed by Strain T99.	S23 serum unabsorbed.	Normal serum.	Broth control.
1:80	0	4	4	4	4	4	4	4	4	4	4	4	4	4	0	0
1:320	0	4	4	4	4	4	4	4	4	4	4	4	4	4	0	
1:1280	0	3	3	3	3	3	4	4	3	4	4	3	2	4	0	

4 indicates complete agglutination and sedimentation; 3, complete agglutination with a little turbidity remaining above it; 2, lesser degrees of agglutination; 0, no agglutination.

RESULTS.

In Table III are shown the results in employing 9 sera to study 27 strains by means of agglutination and agglutinin absorption tests. As a rule, at least 2 rabbits were immunized against each strain; since there was never any significant difference in the behaviour of the 2 sera thus prepared against a single organism they are not represented separately in the table. The results obtained with the 2 tests are shown in parallel columns for purposes of comparison. 2 strains (T10 and T30) isolated from the same patient were serologi-

cally identical: T10 was, therefore, studied in greater detail than T30; the same remarks apply to T25, which was apparently the same as T20.

Several facts are at once apparent on studying the results of the agglutination and agglutinin absorption reactions. All strains were agglutinated by and absorbed agglutinins from their homologous sera; but in several instances there was a failure to obtain both reactions even though one of the two was positive. If it is necessary to have both agglutination and agglutinin absorption positive in order to identify any 2 strains as the same it is obvious that no 2 strains from our patients were identical, with the doubtful exceptions of T20 and T77. This seems to answer our original question; but incidentally a number of other points arose which are of general interest in the study of streptococci.

It is not uncommon to find streptococci which agglutinate with the sera prepared against other strains but which are distinct from those strains by the agglutinin absorption test. Thus Strains T92 and T107 were agglutinated by a number of antistreptococcus sera, but failed to absorb the agglutinins from these sera. On the other hand, the demonstration of agglutinin absorption in the absence of agglutination is unexpected. It was found that a scarlet fever strain (NY5) and at least 4 other strains absorbed the agglutinins from T20 serum, without being agglutinated by this serum, nor was Strain T20 agglutinated by serum prepared against either Strain NY5 or Strain S3. Durand and Sédallian (8) met with the same phenomenon on several occasions in the course of their studies on hemolytic streptococci; like us they also encountered a number of strains which were agglutinated by a given serum and yet failed to absorb the agglutinins from it.

Because of the close relationship found to exist between Strains S23, T10, and T40, careful study was given to them. Strain T10 fermented mannitol; the other 2 did not. Sera prepared against each of these strains agglutinated all 3 members of the group to a titre of 1:1280; hence, by agglutination they appeared to be identical. Each serum, on the other hand, had its agglutinins removed by absorption only with its homologous strain; absorption with the other 2 organisms did not lower the titre appreciably for that homologous

TABLE III.

Summary of Results of Agglutination and Agglutinin Absorption Tests.

Strains from rheumatic fever patients.	Sera against.																	
	T10		T20		T27b		T40		S3		S23		S60		S84		Gordon Type I.	
	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.	Agg.	Abs.
T10	+	AB	0	No	0	No	+	No	0	No	+	No	0	No	0	No	0	No
T20	0	No	+	AB	0	No	0	No	0	No	0	No	0	No	0	No	±	No
T25	0	−	+	AB	0	−	−	−	0	−	0	−	0	−	0	−	0	No
T27b	0	No	0	No	+	AB	0	ab	0	No	0	No	0	No	0	No	−	−
T30	+	AB	0	−	0	−	−	−	0	−	+	No	0	−	0	−	−	−
T40	+	No	±	No	0	No	+	AB	0	No	+	No	±	No	0	No	0	No
T43	0	ab	0	No	0	No	Gr.	No	0	No	0	No	0	No	0	No	Gr.	No
T61	0	No	0	No	0	No	Gr.	No	0	No	0	No	0	No	0	No	0	No
T77	0	No	±	AB	0	ab	0	No	0	No	0	No	0	No	0	No	0	No
T81	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
T88	0	No	+	No	0	No	0	ab	0	No	±	No	0	No	0	No	0	No
T102	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
T105	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
Strains from other sources.																		
S3	0	No	0	AB	0	No	0	No	+	AB	0	No	0	No	0	No	0	No
S23	+	No	0	No	0	No	+	No	0	No	+	AB	0	No	0	No	0	No
S60	0	No	0	No	0	No	0	No	0	No	±	No	±	AB	0	No	0	No
S84	0	No	0	No	0	No	0	No	+	No	±	No	±	No	+	AB	0	No
Gordon Type I	0	No	0	No	0	ab	0	No	0	No	0	No	0	No	0	ab	+	AB
T58	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No
T70	Gr.	No	Gr.	AB	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No

T79	+	No	0	AB	0	No	0	No	0	No	0	No	0	No	0	No	0	No
T92	+	No	±	No	0	No	±	No	0	No	±	No	+	No	+	No	0	No
T95	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No	Gr.	No
T99	0	No	±	No	0	No	0	No	0	No	0	No	0	No	0	No	±	No
T107	+	No	±	No	0	No	0	No	+	No	+	No	+	No	+	No	0	No
NY5	0	No	0	AB	0	No	0	No	0	No	0	No	0	No	0	No	0	No
Erysipelas B3	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No	0	No

In the columns dealing with agglutination (Agg.) + indicates good agglutination up to dilutions of 1:1280 or higher; ±, agglutination in dilutions from 1:100 to 1:640; 0, no agglutination or a slight flocculation not over 1:80. Gr. indicates that the strain was too granular to work with.

In the columns dealing with agglutinin absorption (Abs.) AB means that nearly all the agglutinins were removed; a serum originally active at 1:1280 was inactive or almost so at 1:80. ab indicates that there was no agglutination at 1:1280 but strong agglutination at 1:320; the few results in this group probably belong with the group marked 0. No means no significant absorption; agglutination still occurred at 1:1280. – means that no test was carried out.

TABLE IV.

Table Showing Behaviour of S23, T10, and T40 Sera When Unabsorbed and When Absorbed by Their Homologous Strain.

	Serum dilution.	S23 serum unabsorbed.	S23 serum absorbed by S23.	T10 serum unabsorbed.	T10 serum absorbed by T10.	T40 serum unabsorbed.	T40 serum absorbed by T40.	Normal rabbit serum.	Broth control.
Sera tested against Strain S23.	1:80	3	0	4	0	4	0	0	0
	1:320	4	0	4	0	4	0	0	—
	1:1280	4	0	3	0	4	0	0	—
Sera tested against Strain T40.	1:80	4	0	4	0	4	0	0	0
	1:320	4	0	4	0	4	0	0	—
	1:1280	3	0	3	0	4	0	0	—
Sera tested against Strain T10.	1:80	4	0	4	0	4	0	0	0
	1:320	4	0	3	0	3	0	0	—
	1:1280	3	0	3	0	3	0	0	—

4 indicates complete agglutination and sedimentation; 3 complete agglutination with a little turbidity remaining above it; 0, no agglutination; —, no test made.

TABLE V.

Table Showing that Sera Absorbed by a Heterologous Strain Can Still Agglutinate the Homologous Organism but that This Treatment Removes All Agglutinins for the Absorbing Strain.

	Strains against which sera were tested.											
	Streptococcus S23.				Streptococcus T10.				Streptococcus T40.			
Serum dilution.	S23 serum absorbed by T10.	S23 serum absorbed by T40.	T10 serum absorbed by S23.	T40 serum absorbed by S23.	T10 serum absorbed by S23.	T10 serum absorbed by T40.	T40 serum absorbed by T10.	S23 serum absorbed by T10.	T40 serum absorbed by S23.	T40 serum absorbed by T10.	S23 serum absorbed by T40.	T10 serum absorbed by T40.
1:80	4	3	0	0	4	3	0	0	4	4	0	0
1:320	4	4	0	0	4	3	0	0	4	4	0	0
1:1280	4	3	0	0	4	4	0	0	4	4	0	0

For controls on this experiment see Table IV.

4 indicates complete agglutination and sedimentation; 3, complete agglutination with a little turbidity remaining above it; 0, no agglutination.

strain. By agglutinin absorption, then, each member of the group was distinct. This relationship is, of course, closely comparable with that existing between members of other more carefully studied groups of organisms such as the Salmonellas (*Bacillus paratyphosus* B, etc.). It seems necessary, therefore, to postulate the existence of specific and group antigens in these streptococci, as has been done by F. W. Andrewes in the Salmonellas (9). In order to determine whether 1 group antigen was common to these 3 streptococci the following series of experiments were performed: Each of the 3 diluted sera was divided into 3 lots; 1 portion from each serum was absorbed with Strain S23, 1 with T10, and 1 with T40; each of these absorbed

TABLE VI.

Table Showing Results of Absorbing a Serum with a Heterologous Strain and Then Testing the Serum against the Other Heterologous Strain.

Serum dilution.	Strains against which sera were tested.					
	Streptococcus S23.		Streptococcus T10.		Streptococcus T40.	
	T10 serum absorbed by T40.	T40 serum absorbed by T10.	S23 serum absorbed by T40.	T40 serum absorbed by S23.	S23 serum absorbed by T10.	T10 serum absorbed by S23.
1:80	4	4	4	0	4	0
1:320	1	1	4	0	4	0
1:1280	0	0	0	0	1	0

4 indicates complete agglutination and sedimentation; 1, lesser degrees of agglutination; 0, no agglutination.

sera was then tested for its capacity to agglutinate each of the 3 strains. The results are shown in Tables IV to VI.

Table IV shows that absorption of each serum by its homologous strain removed all the agglutinins from it.

Table V shows that absorption of a serum by a heterologous strain removed all agglutinins for the strain used for absorbing but failed to remove the specific homologous agglutinins; *i.e.*, those for the strain used in preparing the serum. The unabsorbed serum and normal serum controls are recorded in Table IV, hence are not repeated in Tables V and VI.

So far the results are not unexpected; but those in Table VI are more interesting. If the cross-agglutinations among these 3 strains

were due to 1 group antigen common to all 3, absorption of any serum by a heterologous strain should remove the group agglutinins for the other heterologous strain.

But it is evident that when Strain S23 was used for absorbing, all "group" agglutinins were removed, as was expected; on the other hand, when Strains T10 and T40 were used for absorbing, the titre of the serum for the heterologous strains was only slightly reduced; the experiment was twice repeated with similar results. When the absorbing doses used were 10, and even 26 times the ordinary dose employed, still T10 serum absorbed by T40 and T40 serum absorbed by T10 were able to agglutinate S23 almost as well as originally. Streptococcus S23 varied from day to day in the degree to which it was agglutinated by T10 and T40 sera; hence it cannot be concluded that any absorption had occurred in the experiment recorded in the first 2 columns of Table VI, for on the date of this experiment it did not agglutinate any better with unabsorbed sera.

In trying to determine whether each strain contained an antigenic element not shared by either of the other 2, the same sample of each serum was absorbed successively by the 2 heterologous strains. In no case did this procedure affect the titre of the serum against its homologous strain. A strain, S65, isolated by Dochez, Avery, and Lancefield (6) in 1918 and found by them to be a homologue of S23 behaved towards Strains T10 and T40 exactly as did Strain S23, both by agglutination and agglutinin absorption tests.

To interpret the foregoing results it seems necessary to assume that each streptococcus contains a specific antigen and at least one other factor which is common to all. The results shown in Table VI, moreover, make it appear that the complete explanation is somewhat complex. It is possible that we have to deal with more than one group antigen, as seems to be the case with the Salmonellas (10); but this is by no means proven. It is also possible that the 3 organisms under study differ in the relative amounts of group and specific antigens which they contain, or that the situation is complicated by other factors as yet not understood. In any case, it would be premature, after a study of only 3 strains to try to portray graphically their antigenic mosaic, though it is tempting to endeavour to do so. The results obtained, however, indicate that hemolytic streptococci may be antigenically related to each other in a rather complex manner.

It has been shown that the members of the Salmonella groups are diphasic (F. W. Andrewes (9, 10)) because some individuals of each strain possess mainly group antigen while others have almost wholly the specific antigen. It seemed desirable, therefore, to pick individual colonies of the 3 streptococcal strains under study and to see if cultures from each colony behaved alike towards the various sera. Broth cultures from 18 colonies of Strain S23, 15 of T40, and 33 of T10 were tested by agglutination against all 3 sera; all behaved alike within narrow limits. The agglutinin absorption test was not applied to the cultures from these colonies; one cannot, therefore, say definitely that they were antigenically identical, but can only presume that all the colonies picked contained at least one common antigen.

Towards the end of this work we encountered another illustration of the difficulties which beset the worker who studies streptococci. It was necessary to prepare a fresh supply of serum against Strain T10: this was found to have as high a titre against T10 as our original serum; it had, however, only a small amount of agglutinins for Strains S23 and T40 although, as will be shown later, it apparently had good protective power against Strain S23 when tested in mice (see Table VIII). The stock blood broth culture which was kept in the ice box and used in preparing this serum was formerly agglutinated by T10, S23, and T40 antisera in dilutions of 1:1280; on retesting, we found that it still agglutinated to 1:1280 with its homologous serum, to 1:1280 with T40 serum, and to 1:320 with S23 serum. On the other hand some of the original culture which had been preserved by drying in the frozen state was still agglutinated with T40 and S23 sera in dilutions of 1:1280, as of old. It thus appears that variations may occur in the power of a culture to show cross-agglutination and to stimulate the production of group agglutinins even though we were unable by picking colonies to demonstrate the existence of sharply defined phases.

The agglutination and agglutinin absorption tests each considered separately would lead to very different conclusions as to the relationships existing among S23, T10, and T40. It seemed of interest, therefore, to compare the relationships indicated by these 2 methods with those demonstrated by protection experiments in mice. In their study of hemolytic streptococci, Dochez, Avery, and Lancefield

(6), whenever they could raise the virulence of a strain high enough to study mouse protection, were able to confirm by means of protection their classification based on agglutination. They did not

TABLE VII.

Protective Power of S23, T10, and T40 Sera against S23 (First Experiment).

	Doses of streptococci.					
	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.
Unprotected mice.....	†20 hrs.	†40 hrs.	†65 hrs.	†72 hrs.	†65 hrs.	†14 days.
Mice protected by S23 serum.....	S.	S.	S.	S.	S.	S.
Mice protected by T10 serum.....	"	†26 hrs.	†40 hrs.	"	"	"
Mice protected by T40 serum.....	†20 hrs.	†30 "	†30 "	"	"	"

S = survived.
† = died.

TABLE VIII.

Protective Power of S23, T10, T40, and Gordon's Type I Sera against Strain S23.

	Doses of streptococci.						
	0.1 cc.	0.01 cc.	0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
Unprotected mice...	†10 hrs.	†20 hrs.	†22 hrs.	†46 hrs.	†20 hrs.	†22 hrs.	†46 hrs.
Mice protected by S23 serum.....	†20 "	†20 "	S.	S.	S.	S.	S.
Mice protected by T10 serum.....	†22 "	†20 "	"	"	"	"	"
Mice protected by T40 serum.....	†20 "	†20 "	†20 hrs.	†22 hrs.	"	"	"
Mice protected by Gordon's Type I serum.....	†20 "	†20 "	†46 "	†20 "	†46 hrs.	†24 hrs.	†46 hrs.

S = survived.
† = died.

study agglutinin absorption; but some of their old strains were fortunately available for our use. Strains S10 and S100 were found by them to be homologues of S60, Strains S15, S50, and S110 were homologues of S84, and Strain S65 was the same as S23. We ap-

plied the agglutinin absorption test to these strains and in all cases the results confirmed the classification made by these workers. In other words, with the strains they studied the results based on agglutination, agglutinin absorption, and mouse protection are identical. Only 1 of our strains, S23, could be rendered sufficiently virulent by repeated passage through rats and mice to be of value in mouse protection tests; it finally killed mice in doses of 0.000001 to 0.0000001 cc. We employed the same technique for our protection tests as that used by the authors mentioned above: mice were given 0.5 cc. of immune serum intraperitoneally and 24 hours later were inoculated by the same route with the appropriate dose of culture suspended in 0.5 cc. of normal salt solution.

The protocols of 2 experiments are given above.

It will be seen that the homologous serum protected mice against 0.1 cc. of S23 culture in the first and 0.001 cc. in the second experiment; these results are perhaps comparable as the second culture used was more virulent. In the first experiment T10 and T40 antisera each showed some protective power, in that they saved the mice from 0.0001 cc. of culture. In the second experiment, however, T40 antiserum only protected against 0.00001 cc. of culture as might be expected because of the increased virulence of the strain; but in the case of T10 antiserum there was protection equal to that afforded by S23 antiserum. This was unexpected since we used, in this test, a fresh lot of T10 serum which agglutinated Strain S23 very feebly. Serum prepared against Gordon Streptococcus Type I, an organism with which Strain S23 showed no cross-agglutination, had no protective power against the latter strain. This is in agreement with Dochez, Avery, and Lancefield (6), who found no evidence of protection by completely heterologous sera. If we had attempted to classify our 3 strains solely by the mouse protection method there would have been some doubt whether T10 and more doubt whether T40 should be included in the same group with S23.

DISCUSSION.

Our inability to demonstrate a serological relationship among hemolytic streptococci from patients with rheumatic fever needs no further discussion. We realize, of course, that this failure would

be more convincing if we could have obtained more cultures at the time of the onset of the sore throat.

Without preparing many more sera it is not possible to state that all the streptococci studied were serologically distinct; we have not attempted a complete classification of the 27 strains. It is interesting however, to note that we met with no strain in New York City which corresponded with any of the 4 type strains which Dochez, Avery, and Lancefield encountered in Texas or with Gordon's Type I strain, which he believes to include the majority of *Streptococcus pyogenes* (3). Our failure to find strains similar to those isolated in Texas is not surprising in view of the fact that Valentine and Mishulow (11) in New York during 1921 were able to isolate from 52 streptococcus-infected patients only 1 strain identical with any of the 4 type strains of Dochez, Avery, and Lancefield; in their studies both agglutination and agglutinin absorption tests were applied. Krumwiede, Cooper, and Provost (12) have advanced arguments indicating that reciprocal agglutinin absorption is the ultimate criterion of serological likeness of two bacterial strains. Our work indicates that such agglutinin absorption must be used to control the results of agglutination. We have not admitted the identity of any 2 strains unless the results of these 2 tests agree; there seems at present no reason for applying to the classification of the hemolytic streptococci criteria less rigid than are employed with other groups of bacteria. The results obtained with Strains S23, T10, and T40 raise the whole question of the relative value of agglutination, agglutinin absorption, and protection for the classification of streptococci. In spite of much labour the classification of the hemolytic streptococci is in its infancy. It is a study complicated by the fact that, although complement fixation (Kinsella and Swift (13)) and precipitin reactions (Hitchcock (14)) might seem to indicate that the group is a homogeneous one, yet agglutination, agglutinin absorption, and mouse protection methods tend to show that it is heterogeneous. The explanation of the results obtained by the complement fixation and precipitin tests is probably to be found in the existence of identical or closely related nucleoprotein and soluble substance elements in different strains (Lancefield (15); Hitchcock (14)). It seems not unlikely that, as Durand and Sédallian (8) suggest, streptococci do not fall into sharply defined types

but contain an antigenic mosaic, the constituent parts of which are distributed in a more or less complicated manner amongst the members of the class.

SUMMARY.

1. Thirteen strains of hemolytic streptococci isolated from patients with acute rheumatic fever did not show any evidence of being related serologically.

2. Strains of hemolytic streptococci exist which, although identical by the agglutination test, do not show complete reciprocal agglutinin absorption. Sera prepared from such related strains may show some protective power in mice against another member of the group.

3. Some strains of hemolytic streptococci may absorb agglutinins from a serum prepared against another strain, and yet may fail to be agglutinated by that serum.

4. None of 20 strains of hemolytic streptococci isolated in New York City in 1924, and 1925, corresponded serologically with Dochez, Avery, and Lancefield's 4 type strains or with Gordon's Type I strain.

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THE PRODUCTION OF ALBUMINURIA BY RENAL VASOCONSTRICTION IN ANIMALS AND IN MAN.

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The production of albuminuria[1] as the result of obstruction or interruption of blood flow through the kidney by compression of the renal artery was first demonstrated by Hermann (1). Subsequent work has shown that the glomerulus is the structure in which the escape of albumin in this condition takes place (2). Evidence that interruptions occur spontaneously in the circulation through individual glomeruli has been obtained by Richards and Schmidt (3) and the view has been advanced by them that these interruptions occur in such sequence and distribution as to permit simultaneous activity in some and inactivity in other glomeruli. It seems obvious that the interval of normal intermittence of the glomerular circulation cannot be great enough to produce asphyxial damage to glomerular structures; otherwise normal urine would contain albumin. But it was suggested by Richards (4) that if the duration of the intermittent cessations of glomerular blood flow should increase, albuminuria might result under conditions not far removed from the physiological. Since various renal vasoconstrictor agencies have been shown to decrease the number of glomeruli simultaneously open to the circulation (5), it seems permissible to assume that these agencies increase the intervals of glomerular intermittence. Hence the experiments here reported were designed for the study of a possible association of renal vasoconstriction and albuminuria. The results have confirmed our anticipations and have clarified our understanding of the fashion in which certain non-nephritic albuminurias may arise in man.

[1] The word albumin is used in its clinical sense throughout this paper; *i.e.*, protein precipitated from the urine by the usual clinical reagents.

In the older literature observations are recorded which are in harmony with the conception outlined and which may be explained by it. Claude Bernard (6) produced albuminuria by stimulating the renal nerves and by "*piqûre.*" Attracted by the association of hypertension and albuminuria in nephritis, a group of German investigators, Litten, Grützner, Senator, and others, attempted to produce albuminuria by increasing blood pressure. In a review of this literature Senator (7) states (in translation),

"The greatest increase of pressure in the aorta can be produced with ease and precision by electrical irritation of the cervical spinal cord, by producing dyspnœa, by poisonous doses of strychnia, digitalis, etc. All these means have been employed to produce albuminuria and it may be asked with what results? All observers agree in stating that invariably during the rise of the aortic pressure and at its height the flow of urine altogether ceases. Subsequently when, with the falling pressure, secretion is restored and is more copious than before, a (gradually diminishing) excretion of albumin takes place."

The interpretation of these experiments was debated. Senator believed the increased filtration pressure forced the albumin through the glomerulus. Litten (8), who performed the experiment with strychnine, believed that the drug caused a constriction of the renal capillaries which produced ischemia and an albuminuria analogous to that obtained by Hermann after compression of the renal artery.

The Production of Albuminuria by Adrenalin in Rabbits.

A few preliminary experiments were made on rabbits. An unanesthetized animal was placed on a rabbit board and a catheter inserted into the bladder. The urine present was drawn off and then the urine dropped out steadily as it was secreted. The bladder was gently compressed at intervals to prevent urine from accumulating in it.

Adrenalin was injected into an ear vein at a constant rate. At the beginning of the injection the animals became rigid, but they relaxed immediately and remained quiet during the rest of the injection.

Following this albumin appeared in the urine or if previously present it increased in amount. The details are to be found in Table I. Examination of the sediment showed that the albuminuria could not have been caused by bladder trauma.

This series of experiments shows that small doses of adrenalin injected intravenously can produce albuminuria,[2] but they give no clue to the manner in which it is produced.

[2] The albuminuria occasionally observed after subcutaneous injections of suprarenal extract by Blum (9) and Zuelzer (10) is not to be regarded as in the same category with that which is discussed in this paper. Pathological alteration of many tissues including the kidney is known to follow such large doses of adrenalin (11, 12).

Albuminuria Following Renal Vasoconstriction in Dogs.

In order to study more closely the relation of the renal circulation to the production of albuminuria, a series of experiments was performed on anesthetized dogs in which blood pressure, kidney volume, and urine flow were recorded.

Technique.—Healthy dogs were given from 500 to 1000 cc. of water by stomach tube and injected subcutaneously with 0.18 gm. of luminal sodium per kilo 3 hours before operation. Tracheal and carotid cannulas were inserted, the intestines and spleen removed after ligature of their vessels, and the left kidney placed in an oncometer. A cannula was inserted into each ureter close to the kidney and the urine led to Condon drop recorders, placed close to the animal, and collected in test-tubes underneath. The operation was usually completed within 1¼ hours.

TABLE I.

Action of Intravenously Injected Adrenalin in Producing Albuminuria.

No.	Adrenalin injected.	Albumin in urine.		
		Before adrenalin.	First specimen after adrenalin.	Next specimen.
8	3 cc. 1:50,000 in 4½ min.	0	0	0
8	4.1 " 1:25,000 " 14 "	0	+	0
2	5 " 1:10,000 " 5 "	0	++	+
1	5 " 1:10,000 " 6 "	+	+++	+++
3	6.5 " 1:10,000 " 8 "	0	+++	−
7	0.5 " 1:1000 " 3 "	0	++++	−

In a few instances ether was required during the operation. The dogs lay on an electric warming pad.

Blood pressure was recorded by a mercury manometer, kidney volume by a sensitive, calibrated Brodie bellows. Compression of the left renal artery was accomplished by means of a long ligature passed about the artery, both ends of which were threaded through a glass tube. By pulling the thread the artery was drawn against the smooth end of the tube and any required degree of compression could be secured without disturbance of oncometer or ureteral cannula. The capacity of the ureteral cannulas and tubes leading to the drop recorders was as small as was practicable. It was measured and taken into account in recording the circulatory conditions which obtained during the elimination of each specimen of urine examined.

Examination of Urine.—In the early experiments of the series, albumin was detected by the Heller and the heat and acid tests. Later, trichloracetic acid was found to be more satisfactory for the small amounts of urine used. 1 cc. test-tubes

were used, 0.12 cc. of 10 per cent trichloracetic acid being added to 0.03 cc. of urine. The turbidity produced in this mixture was compared with that in a control tube in which the same amount of urine was mixed with 0.12 cc. of distilled water. The natural turbidity of dog urine very largely disappears when it is diluted in this proportion. Tests were made in duplicate.

To guard against error which might be introduced because of differences in concentration of urine samples, specific gravity was determined. The method used was that of Hammerschlag, employing tubes containing mixtures of chloroform and toluene of graded densities, each differing by 0.005 sp. gr. from that next adjacent. In questionable cases, when the albumin reactions of two specimens of different specific gravity were compared, the denser specimen was diluted so that the resulting specific gravity was slightly less than that of the other. This precaution makes it unnecessary to consider changes in concentration of the urine as a factor in the results of the tests for albumin recorded in the tables and charts.

The reaction of each specimen was tested with litmus paper. Sediments were obtained either by centrifugation or standing. All these tests could be performed on a specimen consisting of 0.25 cc.

When scrupulous care was taken to avoid exposure, cooling, or trauma to the kidneys the operations caused no albuminuria in the majority of dogs. Some showed a slight albuminuria which cleared up in 10 or 15 minutes after the operation; in a few the albuminuria persisted throughout the experiment. In a few instances albuminuria developed spontaneously during the course of an experiment and continued until urine flow ceased.

Pathological Examination.—After the experiments the kidneys were examined.[3] All were grossly normal. I am indebted to Dr. Allen J. Smith for the examination of the sections of Dogs 5 to 16 inclusive. He reported them normal with the following minor exceptions. Dog 7 had a few foci of round cell infiltration in both kidneys. An occasional immature nematode surrounded by a tubercle-like capsule was found in the kidneys of Dog 8. The kidneys of Dog 9 had a few small points of hemorrhage in the cortex and pyramids and a small amount of coagulated material (albumin) was seen here and there in the tubules and spaces of Bowman. The kidneys of Dog 15 which received a large amount of saline solution intravenously after the experiment

[3] Albuminuria was produced more than once in all the experiments. In Dog 12 it was produced four times without causing any detectable pathological change in the kidney.

were edematous. The large veins were prominent in all sections but those of Dog 13. The kidneys of Dogs 1 to 5 were not sectioned.

Results.—In our first experiments, the left renal artery was first compressed to such an extent as to cause marked decrease in kidney volume without extinguishing pulsations in the oncometer record. Decreased urine flow and transient albuminuria on the left side occurred. Later 0.4 cc. of 1:5000 adrenalin solution was injected intravenously during a period of 4 minutes, the rate of injection being such as to produce a decrease in volume of the left kidney as nearly as possible identical with that previously caused by arterial compression. The result of the adrenalin injection was studied particularly on the right kidney because this had not been involved in the preceding arterial compression. The effects of adrenalin now exhibited by both kidneys were precisely analogous to those shown by the left under the influence of arterial compression—decrease in urine flow and appearance of albumin in the urine. The results are graphically shown in Chart 1. Similar results have been obtained with adrenalin in four experiments so that we may say with certainty that adrenalin, injected in such amount and at such a rate as to cause decrease in kidney volume and urine elimination, also causes an albuminuria which disappears soon after circulatory conditions have become normal (Chart 2). In experiments (Dog 8) in which albumin was present in the urine before adrenalin was injected, the amount of albumin eliminated following the adrenalin was increased. In these experiments complete disappearance of the albumin did not follow—a fact which may be attributed to the general conditions of the experiment.

In exceptional experiments the injection of adrenalin was followed by swelling of the kidney and increase in urine flow. When this happened no albumin appeared in the urine. In Experiment 13, (see Chart 3) the left kidney swelled and urine from it increased; it contained no albumin. On the other hand the right kidney showed first diuresis and then a period of anuria followed by the elimination of albuminous urine. In this experiment we may believe that vasoconstriction occurred in the right but not in the left kidney. Since albuminuria occurred on the right and not on the left side, we may conclude that this feature in its action is a result of renal vasoconstriction and not a result of direct action of adrenalin on glomerular

membranes. In two other experiments (Nos. 2 and 3) diuresis without albuminuria followed adrenalin action.

Two other means, CO_2 inhalation and hemorrhage, have been used in dogs, prepared as described, for the purpose of producing renal vasoconstriction and both have caused transient albuminuria similar to that following adrenalin. Dog 16 (Chart 4) inhaled 25 per cent CO_2 from a large spirometer for a period of $2\frac{1}{2}$ minutes. Dyspnea was pronounced and kidney volume decreased. Urine, not quite free from albumin before CO_2, became markedly albuminous after it. The albumin content then decreased to its former concentration. This result was verified in two other experiments.

In Dog 15 (Chart 5) 50 cc. of blood was drawn from the femoral artery. Kidney volume and urine flow decreased. After 1 minute the blood was reinjected (no anticoagulant having been used). Urine flow was not reestablished and hence infusions of saline solution were given to start and maintain it (56 cc. in 35 minutes). Transient albuminuria occurred.

In a number of instances urine ceased flowing either during the operation or spontaneously during the course of an experiment. This was regarded as due to spontaneous contraction of renal vessels with consequent diminution in the number of glomeruli open to the circulation. This supposition was verified in two animals by injecting Janus green B into the aorta *via* the carotid artery and examining sections of the cortex of the excised kidney. Many unstained glomeruli were found. In such instances when urine elimination was reestablished by injecting a diuretic (0.9 per cent sodium chloride, 3 per cent pituitrin, or 5 per cent sodium sulfate) the first urine eliminated contained far more albumin than that which had first been collected before suppression and this disappeared as diuresis continued. Such a result is shown in Chart 6. Experiments of this type show that spontaneous as well as experimentally induced renal vasoconstriction is followed by albuminuria.

The Production of Albuminuria by Emotional Excitement in Cats.

Since the previous experiments showed clearly that renal vasoconstriction caused by adrenalin may result in albuminuria, it seemed desirable to make similar tests on the urines of animals which had been

subjected to emotional excitement, because of the well based belief that increased liberation of adrenalin takes place in such a state (13).

Female cats were fastened to a holder and catheterized. This was repeated hourly during the experimental period. During the hour between the second and third catheterizations the cat was annoyed either by the presence of a dog or by repeated weak electric shocks.

The results of a typical experiment are shown in Table II. Urine obtained at the first catheterization, eliminated before any manipulation of the animal, was albumin-free; the second specimen contained a

TABLE II.

The Production of Albuminuria by Emotional Excitement.

Cat B. 2.5 kilos. Urine obtained by catheter. Between 1.45 and 2.30 p. m. the cat was frightened by weak electric shocks. She starts when shocked, growls, pupils are dilated, respiration rapid, hair bristles.

Time.	Vol. of urine.	Albumin in urine.		
		Trichloracetic acid.	Roberts' test.	Heat and acid.
p.m.	*cc.*			
12.30	11.1	0	0	0
1.30	1.7	+	Tr.	+
2.30	1.1	+++	+++	+++
3.30	1.2	++++	++++	++++
4.30	1.15	+++	++++	+++
a.m.				
9.30	2.3	0	0	0

All urine reactions were acid. Sediments contained a few white cells.

trace;[4] that eliminated during the next 3 hours was highly albuminous; and a final specimen obtained next morning was again free of albumin. The sediments were negative. The trace of albumin in the second specimen may be regarded as due to excitement associated with the first catheterization. Tremor and sometimes a short struggle

[4] The larger amounts of urine obtained permitted more tests than in dog experiments. The trichloracetic test (0.2 cc. added to 0.4 cc. urine), the heat and acetic acid, and the ring test with Roberts' reagent were performed. The reaction and specific gravity were determined and the sediment examined as in the dog experiments.

occurred; pulse and respiratory rate increased. In a cat in which, as a result of repetition, catheterization was no longer a source of annoyance no albumin appeared because of it. Similar results to that summarized in Table II were obtained in twelve experiments carried out on five cats. Two of these had albumin constantly in their urine, the annoyance causing an increased concentration of albumin which soon diminished to its former level. One cat (B) in which this albuminuria had been produced five times was killed. Its kidneys were normal on gross and microscopic examination.[5]

In one instance a male cat was confined in a large cage for 6 days, its bladder being emptied each morning by compression. The urine contained no albumin until, on the 4th day, the cat was annoyed for an hour by mild electric shocks. The next specimen obtained contained albumin. No albumin appeared on the following day. The sediments were all negative. This animal was subsequently killed, the kidneys were normal to gross examination. The sections, examined by Dr. Smith, showed normal glomeruli, but there was cloudy swelling of the tubules in some areas. An occasional cast was seen in a tubule and there were several areas of round cell infiltration. It is difficult to regard these abnormalities, so common in cats, as causally connected with the results of the experiment.

In these experiments we have no direct evidence of renal vasoconstriction, but believe it to occur from what is known of the physiology of emotional states. The behavior of the cat, the growling, the starting, the erected hair, and the rapid respiration furnished evidence that the changes associated with emotional disturbances were occurring in the experiments.

The Production of Albuminuria in Man by Ephedrine.

An opportunity for making some analogous observations in man was afforded by the fact that a clinical study of the drug ephedrine, new to this country, was being carried out by Dr. T. Grier Miller (14) in the Medical Division of the University Hospital. The action of this substance, recently studied in animals by Chen and Schmidt (15) resembles that of adrenalin, differing, however, in that its effects are more prolonged and may be reliably elicited by oral or subcutaneous injection. In animals it usually causes constriction of renal

[5] I am indebted to Dr. Morton McCutcheon for the examination of the sections.

vessels; occasionally dilatation and diuresis. There is no evidence that it is intrinsically injurious to the kidney or any other organ in therapeutic dosage.[6] Therefore with the permission and cooperation of Dr. Miller, we studied the urines of patients, who volunteered to receive ephedrine, with the expectation of finding transient albuminuria following its administration in those whose blood pressure rose considerably and whose renal vessels were presumably constricted and not in those in whom blood pressure did not rise.

Male patients, showing no evidence of renal disease, were selected. The majority were convalescent and soon to leave the hospital. They received 100 to 200 cc. of water hourly and voided urine hourly.[7] On the day of the experiment they remained in bed, receiving no food until noon. They lay on a couch during the afternoon. Included in the series are results on two normal men, who fasted till 6 p.m. of the experimental day and who spent most of the day in work in the laboratory.

The results (Table III) have been arranged in three groups. In Group I are included those cases which showed a rise in blood pressure without diuresis; *i.e.*, which gave evidence of renal vasoconstriction. In all of these positive tests for albumin followed the drug, ranging from a heavy turbidity to a flocculent precipitate in the heat and acid tests. The albuminuria ceased after a few hours. Group II includes the cases which showed practically no rise of blood pressure nor significant diminution of the amount of urine after the drug; *i.e.*, which gave no evidence of renal vasoconstriction. A rise of blood pressure of 10 mm. of Hg or less, is not considered significant. Albumin appeared in only one of these cases within 7 hours:[8] in this a very faint trace

[6] Patients at the University Hospital have received ephedrine in full doses daily for a month or more without harmful effect and often with benefit (14).

The plant containing ephedrine has been used in Chinese medicine for centuries.

[7] The urine was tested with Roberts' reagent immediately after voiding, then stored in the ice box till the next day, when the heat and acetic acid and trichloracetic acid tests (0.25 cc. 10 per cent trichloracetic acid and 2 cc. urine) were performed. The latter test is much more delicate than the clinical tests and than in the dilutions in which the reagent was employed in dog and cat experiments.

[8] Case 64 in Table II (page 159) of Dr. Miller's paper (14) is an apparent exception to this statement. This is due to an error of transcription. The rise of systolic blood pressure should have been recorded as 22 mm. of Hg as in Table I of that paper.

occurred 5 hours after administration of the drug.[9] Group III includes cases which showed rise of blood pressure and diuresis after ephedrine. Transient albuminuria was detected in the majority but the concentration of albumin was usually less than in Group I. In some no albumin was found.

TABLE III.

Albumin in Urine of Patients Treated with Ephedrine.

Group.	No.*	Dosage of ephedrine sulfate.	Mode of administration.	Maximum rise of blood pressure.	Ratio rate of urine flow before and after ephedrine.†	Maximum albumin after ephedrine.‡
		mg.				
I	4	100	Subcutaneous.	50	10: 8	++
	8	100	"	45	10: 5	+++
	9	100	"	45	10:10	+++
	14	100	Oral.	40	10:10	+++
	47	100	Subcutaneous.	15	10: 8	+++
II	52	100	"	5	10: 9	Tr.
	59	100	"	8	10:57	0
	77	75	Oral.	3	10:17	0
	C	75	"	10	10:30	0
III	3	100	"	58	10:16	0
	10	100	"	45	10:12	++
	19	100	Subcutaneous.	30	10:22	+
	29	100	Oral.	29	10:36	++
	37	100	"	22	10:24	+++
	A	100	"	35	10:20	Tr.
	B	100	"	40	10:18	0

*Case numbers are those in Dr. Miller's series.

†Amounts of urine eliminated are recorded in Dr. Miller's paper.

‡ Both Roberts' and heat and acetic acid tests were used.

The results in Groups I and II are completely consistent with the expectations based on animal experiments; in Group III the consistency is less apparent. It may be assumed that in cases exhibiting increased blood pressure, diuresis, and no albumin that dilatation of renal vessels occurred as it does in exceptional instances in animals.

[9] Tests of this strength had been occasionally obtained on the urine of this patient by the hospital laboratory before administration of the drug.

In those which showed increased pressure and diuresis with albuminuria it is permissible to think of alternation of constriction and dilatation. The marked fluctuations in urinary output in Cases 19 and 37

TABLE IV.

Case 52. H. H. M. Age 60. Asthma. Had been receiving 50 mg. ephedrine subcutaneously every 6 hours day and night since Mar. 22 with marked diminution in number of attacks. No symptoms on experimental days. On Apr. 6 he received his routine doses of ephedrine sulfate at 9 and 3 o'clock day and night and drank 200 cc. of water every hour. On Apr. 14 100 mg. ephedrine sulfate was given at 9 a.m. instead of the routine dose and he drank 100 cc. of water every hour. When the drug was discontinued his attacks became more frequent and severe.

Time.	Blood pressure.		Vol. of urine.	Sp. gr.	Albumin.		
	Systolic.	Diastolic.			Roberts' test.	Heat and acid.	Trichloracetic.
Apr. 6.			*cc.*				
Noon.			25		0		+
3 p.m.			300	1005	0		0
6			100	1015	0		+
8			250	1008	0		0
10			400	1005	0		0
11.30			325	1005	0		0
6 a.m.			325	1008	0		0
Apr. 14.							
8 a.m.			70	1020	0	0	++
9	110	70	60	1020	0	0	+
10	115	75	60	1018	0	0	+
11	115	70	60	1017	0	0	+
12	115	80	50	1015	0	0	+
1 p.m.	120	75	50	1016	0	0	++
2	122	75	80	1011	Tr.	?+	++
3	95	75	50	1014	0	0	+

All urine reactions were acid. Sediments contain a few epithelial cells and rare white blood corpuscles.

support this interpretation. It seems significant that the albumin in these cases is present in the urines eliminated at the slowest rates.

Detailed reports are given of four cases in Tables IV to VII. Case 52, the asthmatic, H. H. M., received 50 mg. of ephedrine hypodermically every 6 hours for 22 days before the experiment of April 14

TABLE V.

Case 59, M. L. Age 13. Severe diabetic. Sugar-free on insulin. Morning insulin and breakfast omitted; insulin and lunch at 12.30. Drinking 100 cc. water each hour. No symptoms. Received 100 mg. ephedrine sulfate subcutaneously at 9 a.m.

Time.	Blood pressure.		Vol. of urine.	Sp. gr.	Albumin.		
	Systolic.	Diastolic.			Roberts' test.	Heat and acid.	Trichlor-acetic.
Apr. 14.			*cc.*				
8 a. m.			12		0	0	Tr.
9	110	80	70	1019	0	0	"
10	118	75	160	1015	0	0	"
11	110	65	340	1009	0	0	"
12	110	65	200	1010	0	0	"
1 p. m.	102	70	180	1014	0	0	"
2	82	55	240	1007	0	0	"
3	80	55	170	1006	0	0	"
4	90	70	150	1006	0	0	"

Reactions all acid. Sediments contain amorphous and crystalline urates.

TABLE VI.

C. F. S. (No. 19). Age 31; normal. Drinking 150 cc. water per hour and starving all day. Received 100 mg. ephedrine sulfate subcutaneously at 9.30 a.m.

Time.	Blood pressure.		Vol. of urine.	Sp. gr.	Albumin.		
	Systolic.	Diastolic.			Roberts' test.	Heat and acid.	Trichlor-acetic.
Apr. 21.			*cc.*				
8 a. m.			130	1009	0	0	0
9	110	70	140	1006	0	0	0
10*	130	75	305	1007	0	0	0
11	135	80	102	1009	+	?	++
12	125	70	500	1002	0	0	0
1 p. m.	125	70	148	1004	0	0	0
2	124	70	202	1004	0	0	0
3	120	70	260	1004	0	0	0
4	122	75	275	1007	0	0	0

Reactions alkaline. Sediments not examined.

* At 10.30 a. m. systolic blood pressure was 140.

with marked clinical improvement. The failure to produce either rise of blood pressure or albuminuria is evidence that ephedrine *per se* does not injure the kidney. This is also true of Case 59 which received

TABLE VII.

G. H. (No. 8). Age 40. Pleurisy. Tuberculosis (?). Convalescent and without symptoms on day of test. Received 100 mg. ephedrine sulfate subcutaneously at 9 a.m., Apr. 14. No ephedrine on Apr. 15.

Time.	Blood pressure.		Vol. of urine.	Sp. gr.	Albumin.		
	Systolic.	Diastolic.			Roberts' test.	Heat and acid.	Trichloracetic.
Apr. 14.			*cc.*				
8 a.m.			100	1010	0	0	+
9	95	60	110	1010	0	0	+
10	140	80	50	1012	+	0	+
11	120	70	60	1015	+	+	++
12	128	75	40	1007	+++	++	++++
1 p.m.	110	60	30	1006	+++	++	++++
2	105	65	35	1006	+++	++	++++
3	110	66	150	1005	0	0	+
4	118	70	200	1006	0	0	+
5	118	72	60	1011	0	0	+
Apr. 15							
8 a.m.			60	1015	0	0	+
9	85	55	60	1013	0	0	+
10	90	55	50	1014	0	0	+
11	100	65	70	1013	0	0	+
12	90	66	60	1013	0	0	+
1 p.m.	110	62	50	1016	?	0	+
2	105	68	50	1015	0	0	+
3	105	65	40	1019	0	0	+
4	110	72	50	1018	0	0	+
5				1012	0	0	+

Reactions all acid. Sediments negative till noon, Apr. 14. Specimens at 12, 2, and 3 p.m., Apr. 14, show hyaline and light granular casts. Maximum 10–12 per high power field. Specimens of Apr. 15 negative.

the largest dosage per kilo. The report of Case 19 supports the supposition made in the preceding paragraph, and that of Case 8 shows a result strikingly similar to those of our animal experiments.

DISCUSSION.

Four groups of experiments have been described. Intact rabbits, subjected to intravenous injections of adrenalin, exhibited albuminuria. Adrenalin injected into anesthetized dogs causes albuminuria precisely similar in onset and course to that produced by restriction of the arterial blood supply of the kidney: similar results followed CO_2 and hemorrhage. Cats, aroused to fright or anger, exhibit albuminuria. Men injected with ephedrine, which acts like adrenalin, in many instances show albuminuria. Detectable pathological change in the kidneys sufficient to account for the result was sought and not found.

The conspicuous feature common to the renal action of all the agencies tried is vasoconstriction, and evidence was obtained that when vasoconstriction did not occur albuminuria did not result. Hence the results are consistent with the belief that constriction of renal vessels to such a degree as may occur under unusual circumstances in intact animals and man is followed by transient albuminuria, which is not the result of pathological alterations in kidney structure.

According to older views, this phenomenon would be regarded as the result of diminution in the rate of blood flow through all the glomerular vessels: according to the newer view, the one upon which the plan of these experiments was laid, it is regarded as the result of a series of irregular intermittent interruptions in blood flow through the glomeruli, the duration of which is greater than can be borne by the glomerular membranes without asphyxial alteration in their permeability (3).

None of the experiments here described determines the decision between these two possibilities and it is not improbable that both may apply. However, even when urine flow is not abolished, the maximum concentration of albumin appears, not during the period of vasoconstriction but after it. This observation is more easily interpreted by the newer view, according to which large numbers of temporarily damaged glomeruli open simultaneously when vasoconstriction relaxes.

Allusion has already been made to older experimental results which appear to be completely analogous to those described here: stimula-

tion of renal nerves, *piqûre*, stimulation of the spinal cord, dyspnea, digitalis, or strychnine convulsions—all of these have been shown to be followed by albuminuria. The explanation we have advanced, based upon Hermann's classic experiment and upon new observations on the renal circulation, may be applied to these.

This conception also gives a satisfactory explanation of the albuminuria so often observed in healthy laboratory animals which may be frightened by the method of obtaining urine or other unnatural conditions of their environment. This makes it difficult to interpret experiments in which albuminuria has been produced by means which might excite the animals, as by tying them in extreme lordosis (16) or when urine has been obtained by catheterization of an untrained animal. The albuminuria which is produced by manipulating the kidney through the abdominal wall (17) may also fall into this group, for vasoconstriction promptly follows any rough handling of the kidney in animal experiments.

Of greater interest is the fact that in a variety of conditions frequently associated with albuminuria, encountered in man, it is possible to discern as the chief causative factor restriction of the renal circulation as we have conceived it to occur in these experiments.

In one group of these may be mentioned the albuminuria which is observed to follow unusual emotional disturbance in some normal persons (18)—a phenomenon apparently completely comparable with our observations on frightened cats. Statistics show that about 5 per cent of any large group of healthy people exhibit albuminuria at time of examination (19): associated "nervousness" or "vasomotor instability" has been observed in many of these instances, together with failure of albuminuria to appear in subsequent examinations when the subject is accustomed to the procedure. We think also of "reflex" albuminurias (20) as from irritation of ureter or bladder or other irritative foci as in this category.

That transient albuminuria follows severe muscular exertion has been known for years (21). High blood pressure, mental excitement, and diminution in urine in this condition may be regarded as evidence of renal vasoconstriction.

Albuminuria following convulsions in man is common; associated excitation of centres controlling renal vessels may well be a factor in its causation.

The most commonly accepted theory of the cause of orthostatic albuminuria includes circulatory disturbance of the kidney (22). This was suggested by the symptoms of "vasomotor instability" which many of these patients exhibit; *i.e.*, faintness, flushing, cold extremities, and nervousness (23). Spasmodic constriction of renal vessels was thought by Porges and Pribram (24) to be responsible

because of instances in which the albuminuria cleared up when diuresis was produced by water, coffee, potassium acetate, or hot baths. Almost all observers (24, 25) find that while the patient is standing and albuminuria is present, the rate of urine elimination is decreased. The theory cannot be regarded as proved.

It is highly probable that changes in the renal circulation of the sort we are discussing occur during the induction and maintenance of ether or chloroform anesthesia and that albuminuria following anesthesia is partly at least a result of these. Excitement, partial asphyxia, increased adrenalin liberation, and partial or complete suppression of urine are not infrequently features of anesthesia and may be regarded as responsible for or evidence of renal vasoconstriction. Kemp's experiments (26) upon the connection between asphyxia and ether anesthesia are apropos in this connection.

In cardiac incompetence it is generally believed that maintenance of blood pressure is the result of stimulation of vasoconstrictor centres as a result of their relative anemia. It is highly probable that the renal nerves are involved and that the association of albuminuria with cardiac disease is, in the absence of renal lesions, frequently to be explained by vascular change of the type here discussed.

Without attempting completeness of a catalogue of conditions in animals or in man in which renal vasoconstriction may occur, we believe the evidence is such as to warrant emphasis on the importance of this as a cause of albuminuria; and, in addition, the experiments cited provide a reason for anticipating the appearance of albumin in the urine whenever profuse urine elimination is reestablished after a period of relative or complete suppression which is the result of constriction of vessels of the kidney.

SUMMARY.

Experiments are described which show that:

1. Transient albuminuria follows the infusion of small doses of adrenalin into rabbits, if prolonged over several minutes.

2. Agents causing renal vasoconstriction also cause transient albuminuria in anesthetized, eviscerated dogs.

3. Cats eliminate albumin after having been frightened or enraged.

4. The drug ephedrine, which commonly causes renal vasoconstriction in animals, has been tested in man. Transient albuminuria

followed its administration in those cases in which signs of renal vasoconstriction appeared; it did not in those cases in which such signs were lacking. Evidence was obtained that ephedrine is not intrinsically injurious to the kidney.

The observed facts may be explained by the conception that renal vasoconstriction causes increase in the duration and extent of the normal intermittent interruptions in the glomerular circulation. Permeability of the glomerular membranes is so increased by these lengthened interruptions that when blood flow is reestablished, albumin escapes.

It is believed that this conception may apply to those albuminurias in man which result from excessive muscular exercise or emotion or which are produced reflexly. It may explain the occurrence of albumin in the urine of normal people and in healthy laboratory animals; it may play a part in the explanation of the albuminuria which occurs after anesthesia or in cardiac decompensation. Its possible relation to orthostatic albuminuria has been discussed.

I am indebted to Professor A. N. Richards for the suggestion which led to this investigation and for assistance and advice during the work.

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Explanation of Charts.

Charts showing the effects of various alterations in conditions upon the blood pressure, kidney volume, and urine of anesthetized, eviscerated dogs.

For the sake of clearer demonstration of detail the length of the time unit on the abscissa during the experimental periods between the dotted lines is ten times as great as during the fore and after periods.

Vertical divisions of the urine records together with the numbers under them indicate the division of the urine as collected into samples for testing.

Differences in albumin content are represented by different degrees of cross-hatching of the blocks representing the urine specimens.

Specific gravities are indicated by lines under or in the blocks representing urine.

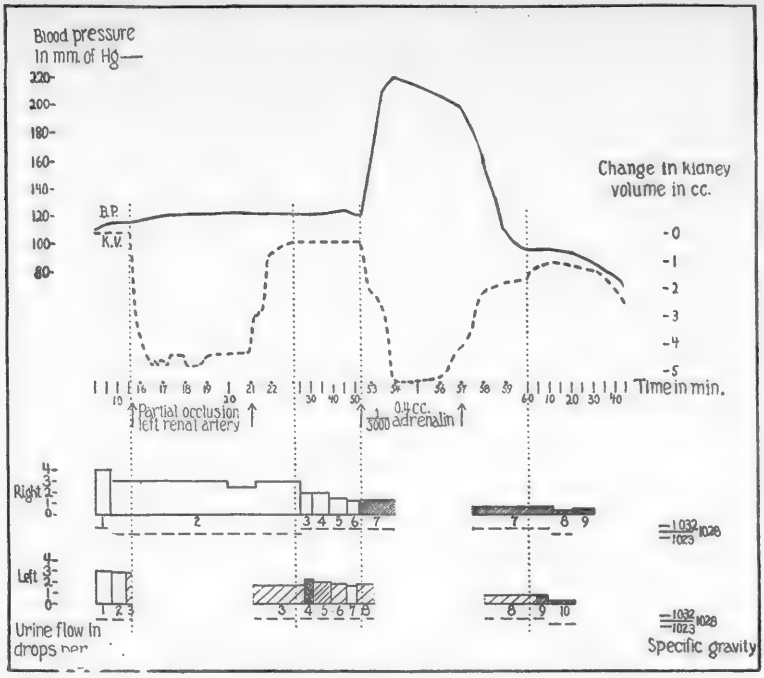

CHART 1. Dog. 9. Comparison of the effects produced by partial occlusion of the renal artery with those caused by intravenous injection of adrenalin. All urines were alkaline in reaction and yielded sediments containing phosphates and a very few epithelial cells.

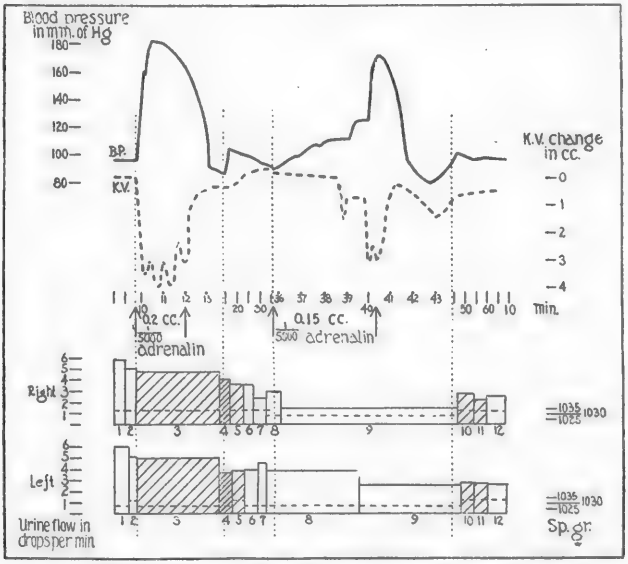

CHART 2. Dog 12. Effects of the vasoconstrictor action of adrenalin. All urine specimens were acid and yielded sediments containing amorphous and crystalline material. Specimens numbered 4 from each kidney contained an occasional hyaline cast.

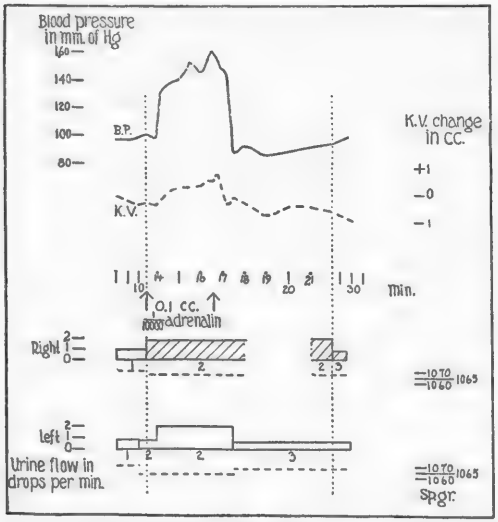

CHART 3. Dog 13. Effects of adrenalin. No vasoconstriction of the left kidney. Probable vasoconstriction of the right. All urines were acid in reaction and yielded amorphous sediments.

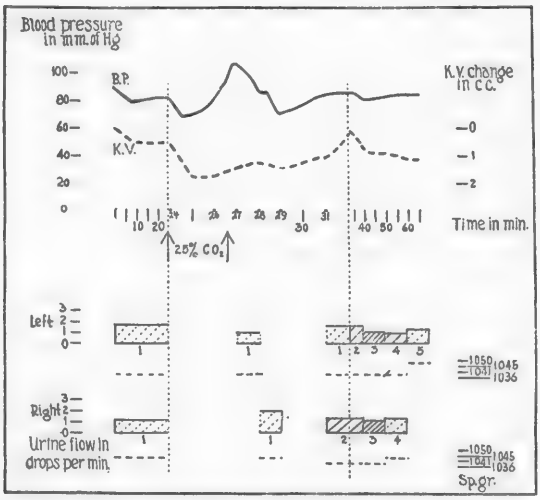

CHART 4. Dog. 16. Effects of inhaling 25 per cent CO_2. All urines were acid in reaction and all contained a few crystals and a small amount of amorphous material.

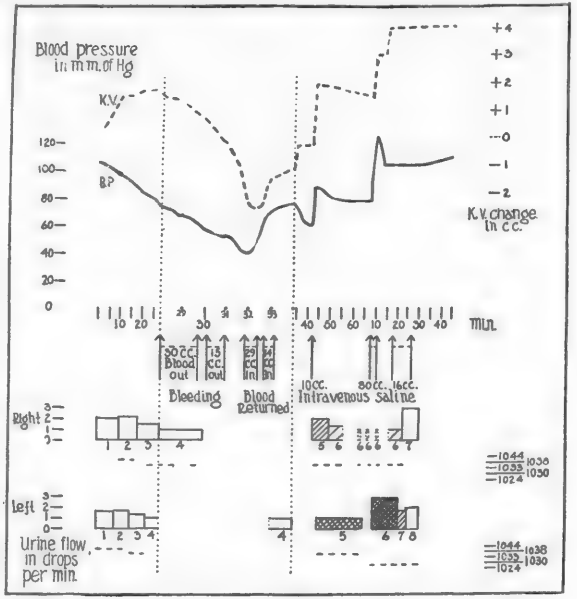

CHART 5.

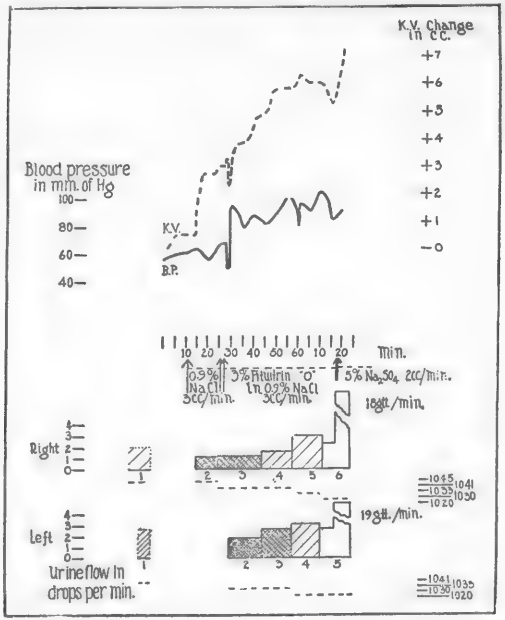

CHART 6.

CHART 5. Dog. 15. Effects of hemorrhage followed by saline infusion. Urine specimens numbered 1 to 5 on both sides were acid: the rest were alkaline. All contained a few crystals and a small amount of amorphous material.

CHART 6. Dog 7. Reestablishment of urine flow, following spontaneous anuria, by injection of diuretics. The rates at which specimens numbered 1 were secreted were not determined.

All urines were alkaline in reaction with the exceptions of No. 1 (left) and No. 2 (right).

The sediments of Specimens 1 (right) and 4 and 5 (left) contained no casts. Many hyaline casts were found in Specimens 2, 3, and 4 (right) and 1, 2, and 3 (left) and light granular casts in Specimen 2 (right). Specimens 5 and 6 (right) contained only an occasional hyaline cast.

CONCERNING THE STABILITY OF THE ACID-BASE EQUILIBRIUM OF THE BLOOD IN PREGNANT ANIMALS.*

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(Received for publication, October 8, 1925.)

In previous publications[1-4] studies have been undertaken which had as their object an investigation of the stability of the acid-base equilibrium of the blood in animals of different age periods. These studies were, furthermore, concerned with the ability of the physiological mechanism which maintains this equilibrium to stabilize it when it was subjected to the strain of certain intoxications and prevent this fundamental physicochemical balance of the organism from being interfered with. In one[1] of these investigations it was shown that as the age of the organism increased, there was a decrease in the stability of the acid-base equilibrium of the blood. Such animals became more susceptible to the toxic effect of uranium nitrate and the general anesthetics.

The present study is concerned with the stability of the acid-base equilibrium of the blood in pregnant dogs at different periods of gestation and in different age groups, as contrasted with non-pregnant animals of the same age periods.

During the past 10 years 96 pregnant dogs have been studied in this laboratory in connection with various purposes. The observations recorded in this paper have gradually accumulated during this period. These animals varied in age from 11 months to 10 years and 4 months. The duration of pregnancy varied in the respective animals from 3 to

* Aided by a grant from the Ella Sachs Plotz Foundation.

[1] MacNider, W. deB., *J. Exp. Med.*, 1917, xxvi, 1.

[2] MacNider, W. deB., *Science*, 1917, xlvi, 643.

[3] MacNider, W. deB., *J. Exp. Med.*, 1918, xxviii, 501.

[4] MacNider, W. deB., *J. Exp. Med.*, 1918, xxviii, 517.

10 weeks. The average duration of gestation in the dog is 9 weeks. Of these 96 animals, 22 have been eliminated from consideration in this study either on account of a lack of information concerning their age, or for the reason that the duration of gestation was not accurately known. The 74 pregnant animals included in this study may be divided into three groups on the basis of their age.

Group I is represented by twenty-two animals between the ages of 11 months and 3 years; Group II by thirty-four animals between 3 and 6 years of age; and Group III by eighteen animals between the ages of 6 years and 10 years and 4 months. The latter group is referred to in this paper as the senile group. In these groups are found animals that have varied in the duration of their pregnancy of from 3 to 10 weeks.

Observations on 68 normal, non-pregnant dogs serve as controls. These animals varied in age from 8 months to 11 years.

Technique.

Both the control and pregnant animals were kept in metabolism cages during each of the six periods of observation. The diet consisted of bread, butter, milk, and a small amount of cooked meat. No restriction was placed on the water intake by the animals. The dogs were catheterized once a day and the urine examined for albumin, glucose, and ketone bodies. The immediate functional capacity of the kidney was determined by the phenolsulfonephthalein test of Rowntree and Geraghty.[5] Determinations of the alkali reserve of the blood (R. pH) were made by the method of Marriott,[6] and these determinations were employed as an index of the acid-base equilibrium of the blood. The above mentioned observations on renal function were made in this study in order to eliminate any renal injury which might be responsible for a disturbance in the acid-base balance of the animals.

Control Observations.

Studies were made on 68 non-pregnant animals. The diet for these animals was the same as for the pregnant animals. Twenty-eight of these dogs forming Group I varied in age from 8 months to 3 years. Twenty-four animals in Group II were between 3 and 6 years of age, while sixteen animals forming Group III were between 6 and 11 years of age. All of the animals of Groups I and II had urines that were free from albumin, glucose, and ketone bodies. Casts were not present.

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[5] Rowntree, L. G., and Geraghty, J. T., *J. Pharmacol. and Exp. Therap.*, 1909, i, 579.

[6] Marriott, W. McK., *Arch. Int. Med.*, 1916, xvii, 840.

The elimination of phenolsulfonephthalein by the respective animals varied from 68 to 82 per cent. There was no evidence of a disturbance in renal function.

The reserve alkali of the blood was determined on 6 different days for each animal. The readings varied within the normal of from 8.0 to 8.15. In animals of such age groups, free from renal disease, the acid-base equilibrium of the blood shows a constant normal balance.

Sixteen of the control dogs, Group III, varied in age from 6 to 11 years. Two of these animals, Observation 32, 9 years and 2 months old, and the animal of Observation 60, 11 years old, showed evidence of renal disease. The urine contained a trace of albumin and casts. The elimination of phenolsulfonephthalein was 48 and 53 per cent by the respective animals. The reserve alkali of the blood for the animal of Observation 32 was 7.9, and for the animal of Observation 60, 7.95. With the evidence of impaired renal function shown by these animals, it is assumed that the disturbance in the acid-base equilibrium of the blood was due to a chronic nephropathy and should be looked upon as a retention phenomenon.

Two dogs of this old group of animals (Group III), Observations 18 and 41, had a normal urine. The elimination of phenolsulfonephthalein by the animal of Observation 18 was 74 per cent, and 69 per cent by the animal of Observation 41. These animals were studied over periods of 2 months. Fourteen reserve alkali determinations were made on each animal, and such observations uniformly showed a disturbance in the acid-base equilibrium of the blood.

The readings for the animal of Observation 18 varied from 7.85 to 7.95, while those for the animal of Observation 41 varied from 7.9 to 7.95. On two occasions the reserve alkali was 8.0. In these two senile animals with an absence of any functional evidence of renal disease there had developed a disturbance in some mechanism other than the kidneys which rendered the animals unable to maintain a normal balance in this physicochemical state of the blood.

The following conclusions are made for the control group of 68 non-pregnant dogs falling in the various age groups.

1. The young and adult females, Groups I and II, in the absence of renal disease constantly maintain a reserve alkali of the blood which is normal and which varies from 8.0 to 8.15.

2. The senile group of animals, Group III, with four exceptions, also maintained a normal alkali reserve of the blood. The reduction in the reserve alkali of the blood in two of these animals can be explained on the basis of a retention phenomenon secondary to renal disease. In the remaining two animals there was no evidence of renal disease. The lack of stability in the acid-base equilibrium of the blood in these animals was due to some other unknown factor.

Observations on Pregnant Animals.

74 pregnant dogs furnished the material for this study. The duration of gestation in the different animals varied from 3 to 10 weeks. These animals, as has been previously stated, were classified into three groups on the basis of their age. In all of the groups the observations recorded here were made either at the end of the first 4 weeks of gestation or within a week of the termination of gestation. A technique similar to that employed for the control groups of animals was used.

Group I.—Twenty-two dogs from 11 months to 3 years of age are included in this group. At the end of the 1st month of the gestation periods all of the animals had a normal urine, an elimination of phenolsulfonephthalein that varied from 68 to 82 per cent, and a reserve alkali of the blood that was normal and varied from 8.0 to 8.15. During the 8th week of the pregnancy of the animal of Observation 10, 3 years of age, and the 9th week of the pregnancy of the animal of Observation 8, 2 years and 9 months of age, the reserve alkali of the blood showed a reduction to 7.95. At this time the urine was normal and there was no reduction in the elimination of phenolsulfonephthalein. The remaining twenty dogs of this group showed no evidence of a disturbance in the acid-base equilibrium of the blood throughout the duration of the pregnancy.

Group II.—Thirty-four dogs are included in this group. The animals varied in age from 3 years and 1 month to 6 years. At the end of the 1st month of the gestation periods all of the animals had a normal urine and a normal elimination of phenolsulfonephthalein which varied from 68 to 76 per cent. All of the animals with one exception had a normal alkali reserve of the blood. The readings varied from 8.0 to 8.15. The animal of Observation 23, 6 years of age, at this stage of gestation had on 6 different days a reserve alkali of the blood of 7.9. The acid-base equilibrium of the blood was definitely disturbed. During the 8th week of the animal's pregnancy there developed an air-hunger type of breathing and associated with this a reduction in the reserve alkali of the blood to 7.8. At this time the animal was forming 442 cc. of urine in 24 hours. There was no albumin in the urine. The elimination of phenolsulfonephthalein was 68 per cent. On the following day the pregnancy came to a natural termination. On the 4th day following this the reserve alkali was 8.05.

During the 8th and 9th weeks of the pregnancies in the animals of Group II the following observations were made. Twenty-two of the animals came to the termination of the period of gestation with a normal urine, an elimination of phenolsulfonephthalein which was not below the minimum normal reading of 68 per cent, and reserve alkali determinations which were within the normal and varied from 8.0 to 8.1. Twelve of the animals gave evidence of a departure from the normal during these late weeks of the gestation period. Three of the twelve animals developed an albuminuria with casts. The amount of albumin in the

urine in these animals was 2.3 gm. for the animal of Observation 6; 2.0 gm. for the animal of Observation 5; and 0.4 gm. for the animal of Observation 13. The elimination of phenolsulfonephthalein was reduced in all of the animals from the normal output of 82, 69, and 70 per cent in the respective animals to 39, 41, and 28 per cent. The reserve alkali of the blood was reduced from the normal of 8.15 in the animal of Observation 6 to 7.9; in the animal of Observation 5, from 8.15 to 7.95; and in the animal of Observation 13, from 8.0 to 7.9. In these three dogs there was definite evidence of renal disease, which might explain the disturbance in the acid-base equilibrium of the blood.

The remaining nine animals in this group of twelve pregnant animals which gave evidence of a departure from the normal during the last 2 weeks of the gestation period had a normal urine and a normal elimination of phenolsulfonephthalein. All of these animals had developed a reduction in the reserve alkali of the blood which varied from 7.85 in the animal of Observation 22, 6 years of age, to 7.95 in the animal of Observation 11, 3 years and 2 months of age.

The animals of Group II, 3 to 6 years of age, have shown during the period of gestation two types of departures from the normal. Both types are associated with a disturbance in the acid-base equilibrium of the blood. The first type, represented by three animals in which such a disturbance occurred, can be explained as developing secondary to renal disease. The second type, which is characterized by a similar depletion in the reserve alkali of the blood, is not associated with any evidence of renal injury and is found to occur in the older members of the group.

Group III.—Eighteen dogs between 6 years and 10 years and 4 months of age are included in this group. During the 4th and 5th weeks of gestation fifteen of these animals had a normal urine and a normal elimination of phenolsulfonephthalein. The output of the dye in a 2 hour period varied from 69 to 81 per cent. Of this number eleven of the animals had a reserve alkali of the blood which was reduced below the normal and which varied from the maximum reduction to 7.9 in the animal of Observation 54 to the minimum reduction to 8.0 in the animal of Observation 20. The remaining four animals of the fifteen which had a normal urine and no reduction in the elimination of phenolsulfonephthalein failed to show any depletion in the reserve alkali of the blood. The reserve alkali determinations for these animals were respectively 8.05, 8.05, 8.1, and 8.15.

Three of the eighteen animals of Group III, Observations 3, 61, and 34, showed the presence of albumin, casts, and diacetic acid in the urine and had a decrease in the elimination of phenolsulfonephthalein to 38, 17, and 41 per cent in the different animals. In these animals with definite evidence of renal disease there was also a reduction in the reserve alkali of the blood. In the animal of Observation 3, the reserve alkali of the blood was reduced from 8.1 to 7.95; in the animal of Observation 61, from 8.15 to 7.9; and in the animal of Observation 34 from 8.15 to 8.0. One of these animals, Observation 3, during the 7th week of gestation developed convulsions and died. No observations had been made within 2 weeks

prior to the animal's death. At autopsy the kidneys showed marked epithelial degeneration.

The animal of Observation 54 which had during the 5th week of gestation a reduction in the reserve alkali of the blood to 7.9, with a normal urine and normal elimination of phenolsulfonephthalein, gave birth prematurely to seven puppies during this week. The animal was the oldest member of the group, 10 years and 4 months old.

The remaining sixteen animals of Group III representing the old animals of the series, all of which have shown a reduction in the reserve alkali of the blood, either with or without evidence of renal disease, completed their period of gestation. In fourteen of these animals there was no further depletion in the reserve alkali of the blood beyond the reading recorded during the 5th week of gestation. In two of the animals, Observations 20 and 57, the reserve alkali underwent a secondary reduction during these last weeks of gestation. In the animal of Observation 20 the reserve alkali was reduced from 8.0 to 7.9 and in the animal of Observation 57 from 7.95 to 7.85.

Excluding those pregnant animals with evidence of renal disease, which may be assumed to have induced the recorded disturbance in the acid-base equilibrium of the blood, the factor of the age of the organism in influencing the stability of this equilibrium is shown as follows by the animals when grouped according to age.

Group I was represented by twenty-two animals that varied in age from 11 months to 3 years. At the end of the 1st month of gestation all of these animals had a normal reserve alkali of the blood, a normal urine with a normal elimination of phenolsulfonephthalein. At the termination of the gestation period in this group of young animals, only two members of the group showed any reduction in the reserve alkali of the blood. These animals had a reserve alkali of the blood of 7.95. The urine was normal, and there was no reduction in the output of the dye.

Group II was represented by thirty-four animals that varied in age from 3 years and 1 month to 6 years. By the end of the 1st month of gestation one of these animals had a reduced reserve alkali of the blood. By the end of the 8th or 9th week of gestation in this group of older animals nine showed a disturbance in the acid-base equilibrium of the blood without any associated renal injury.

Group III was represented by eighteen senile animals, varying in age from 6 years to 10 years and 4 months. During either the 4th or 5th week of gestation in this oldest group eleven of the number had

a reduction in the reserve alkali of the blood which persisted throughout the latter weeks of gestation. None of these animals gave evidence of renal disease. By the end of the period of gestation, fourteen of the eighteen animals in this senile group, in which there was a normal elimination of phenolsulfonephthalein and a normal urine, showed a disturbance in the acid-base equilibrium of the blood.

This summary indicates that as pregnant animals advance in age the stability of the acid-base equilibrium of the blood decreases, and furthermore, that this instability is not due to coincident renal disease.

CONCLUSIONS.

1. The acid-base equilibrium of the blood as indicated by determinations of the reserve alkali of the blood remains constant in non-pregnant animals at different age periods. This statement does not imply that the acid-base balance of such animals at different age periods is a stable balance.

2. In pregnant animals of the same age periods as the control animals there may develop an instability of this equilibrium which is either associated with the occurrence of a renal injury and which may be looked upon as a retention phenomenon, or arises independently of such an injury. In this latter group of animals the disturbance in the equilibrium increases in frequency and is earlier in its appearance in the gestation period as the age of the organism increases.

3. In old and in senile pregnant animals some physiological mechanism other than that of the kidney becomes unable to stabilize the acid-base equilibrium of the blood with the result that as the pregnancy advances this physiological state of the organism (gestation) becomes pathological.

STUDIES ON X-RAY EFFECTS.

XIV. The Effect of X-Ray on the Division Rate of Paramecium.

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Reports on the biological action of x-ray have been contradictory owing largely to the fact that, in the absence of a unit of dosage, accurate comparison of results has been impossible. Furthermore, it has generally been considered that dosage could be disregarded as it was to be expected that larger doses would have simply a more pronounced effect than small ones. As a matter of fact, this idea is erroneous for it has been shown that doses of different intensity may have diametrically opposite effects on a tissue (Murphy and coworkers).

With the development of an x-ray apparatus capable of delivering rays of a constant quality and intensity (Clark), a major difficulty has been eliminated. For the purposes of the present work, paramecium was selected as the biological material because its rapid multiplication renders possible the numerical expression of any reaction to radiation. This selection was made in spite of the fact that no protozoon or simple metazoon has thus far been demonstrated to give any certain response to x-ray exposures. But the ease with which such forms can be handled in large numbers either in mass cultures or individually and the facility with which they may be subjected to a variety of conditions have seemed to make it worth while to investigate anew their response to radiation.

Since reproduction is one of the most easily affected vital functions, we have chosen to test the action of various doses of x-ray on the division rate of these unicellular organisms.

Material.—Two races of paramecium have been utilized; *i.e.*, *Paramecium caudatum* and *Paramecium multimicronucleatum*. For brevity, the former will hereafter be called Race C and the latter Race M. In general appearance they

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are similar although Race M is the larger. Race C has but one micronucleus whereas Race M has four; and Race C has two contractile vacuoles while Race M has from two to seven, depending on environmental conditions (Hance, 1917). Although the paramecia used in the experiments came originally from two pure lines, in many of the experiments the additional precaution was taken of starting new lines from single individuals in order to insure a uniform physiological make-up.

About 15,000 animals were observed individually and many times that number were studied in mass cultures.

Method.—A standard hay infusion was made daily, with 0.5 gm. of hay in 100 cc. of spring water. The mixture was boiled for 5 minutes. For containers, Syracuse watch-glasses boiled in water containing a trace of paraffin were used for the small mass cultures or the isolated individuals. The thin film of paraffin on the glass resulting from this treatment prevented the medium from adhering to the sides of the dishes and rendered easier a complete survey of the animals in the dish.

In order to eliminate the secondary radiation from the glass container in the preliminary experiments, the paramecia were placed in 1 or 2 inch sections of paper soda straws one end of which was plugged with paraffin. With a slender tipped pipette it was possible to place a single individual in such a tube and to recover it without difficulty after the exposure to x-ray. With one end of the straw stopped the surface tension was sufficient to hold the liquid in place during the manipulation. The radiated animals were seldom in the straws more than 1 hour and the absence of any deleterious effects arising from the sojourn therein was shown by the fact that controls allowed to remain inside for 2 days were apparently unharmed. When it became evident that no considerable differences were to be found as far as the rate of division was concerned, the tube was discarded for the simpler one of exposing the animals in the watch-glasses.

The X-Ray Outfit.—The x-ray outfit used in this work has been described (Clark). A broad focus Coolidge tube mounted in a lead-lined cabinet and kept cool by means of a fan is operated on 60 cycle current rectified by kenetrons. Ionization measurements made with apparatus previously described (Clark) have shown that the outfit may be kept in operation continuously without a variation of current or voltage exceeding 2 per cent. In the experiments the rays were produced at 30 kilovolt peak and 22 milliamperes, filtered through very thin cardboard, and used at a target distance of 25.5 cm. Under these circumstances the rays produced, according to our measurements, about 6×10^{12} pairs of ions per gm. per second in air.

EXPERIMENTS.

Experiment 1. To Test the Effect on the Division Rate of Single Exposures to X-Ray.—

(*a*) Samples of the two races of animals were placed in soda straw tubes and exposed to the standard dose used in these experiments for 1, 2, 5, 10, and 20

minutes. One-half of the controls were also kept in tubes for the duration of the exposures and the other half were kept in watch-glasses. After exposure five animals from each batch were placed in five individual watch-glasses and the division rate noted for 5 days. No difference in the division rate of the two sets of controls was observed. The samples of both races exposed for 1 and 2 minutes showed no reaction, while the division rate of those subjected to the same treatment for 5, 10, and 20 minutes was slightly lowered for 2 days, after which it slightly exceeded that of the controls. At the end of 5 days the average number of divisions per day was:

	Race C.	Race M.
Control	0.48	0.6
X-ray (all exposures)	0.46	0.6

No differences in the effects of the various lengths of exposure were noticed and the total number of divisions of both x-rayed and control lines were the same at the end of 5 days as shown above. The tests involving 10 minute exposures have been repeated three times, and the 20 minute exposures eight times always with similar results. See Charts 1 and 2, Graphs 1 and 6.

(*b*) In all of the following experiments mass cultures of the two races of paramecium were first exposed in watch-glasses. Then from each exposed culture from ten to twenty-five animals were isolated in individual watch-glasses and the division rate followed for from 5 to 15 days. The average rate of division was obtained by dividing the total number of animals found in all of the watch-glasses by the number of individuals originally isolated. In this series of experiments exposures lasting 1, 1½, 2, 3, 4, 5, and 6 hours were used. Exposures up to 3 and 4 hours long failed to produce any more marked or different effects on the division rate than those lasting less than 1 hour but after the 5 and 6 hour treatments the division rate was slightly increased over that of the controls. As the results seemed to be complicated with temperature the detailed account is omitted here to be reported when more complete data are available. With the exception of the 1½ hour period the other exposures have been repeated from two to eight times. See Charts 1 and 2, Graphs 2, 3, 4 and 7, 8, 9 for a diagrammatic representation of the results reported above.

As the protocols show, paramecia were exposed to a range of the x-ray for periods of from 1 minute to 6 hours. Even the largest dose proved insufficient to cause the death of the animals. It was observed, however, that there was a slight but constant depression in the division rate lasting from 2 to 4 days and occasionally for 5 days. After this period the x-rayed lines divide slightly faster than the controls. This

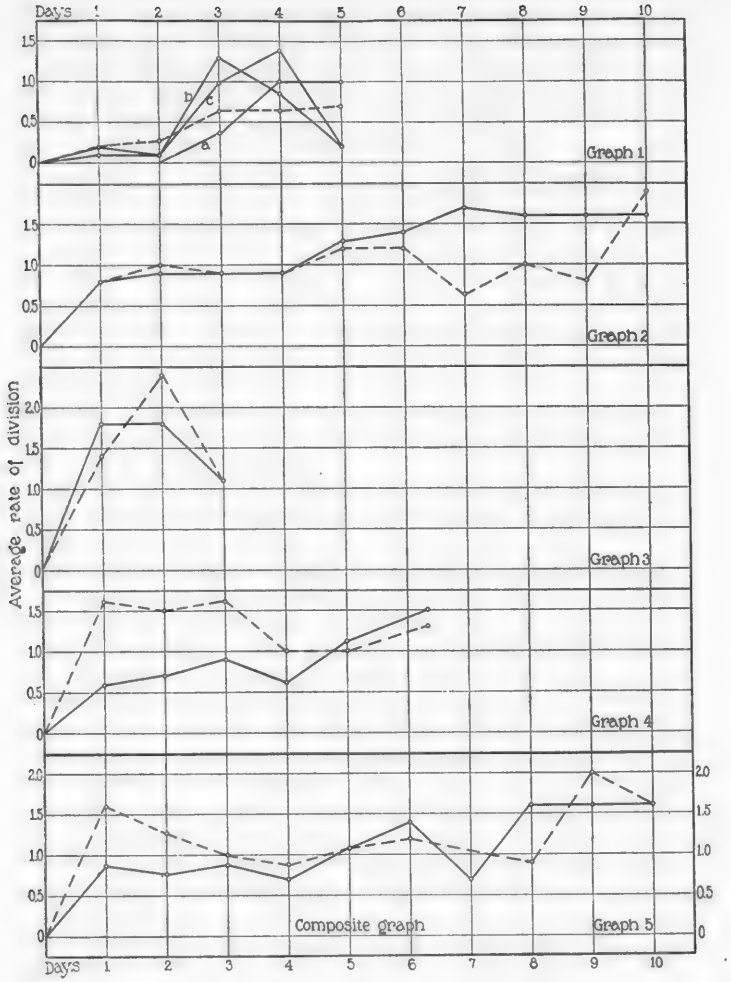

CHART 1. Graphs illustrating the effect of x-rays upon the division rate of *Paramecium caudatum*. The rates of division in tenths of a division are plotted vertically, while the number of days following exposure to x-ray are given horizontally. In all the graphs the broken lines represent the division rate of the control or non-radiated paramecium, the unbroken lines the radiated forms. Graph 1. Curve *a*, 5 minute exposure; curve *b*, 10 minute exposure; curve *c*, 20 minute exposure. Graph 2. 1 hour exposure. Graph 3. 2 hour exposure. Graph 4. 4 hour exposure. Graph 5. Composite graph made by averaging the figures upon which the first four graphs are based.

acceleration was about sufficient to offset the depression so that after awhile the x-rayed and control strains had produced about the same

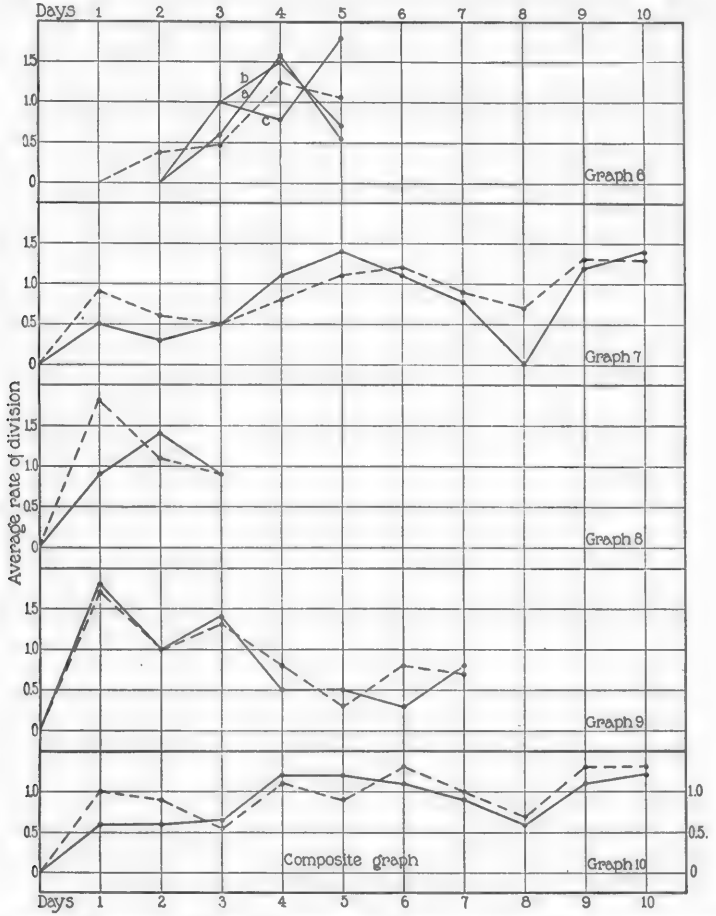

CHART 2. Similar to Chart 1 except that the reactions of *Paramecium multimicronucleatum* to x-ray are illustrated. Symbols and descriptions as in Chart 1, Graphs 6 to 10 being comparable in order to Graphs 1 to 5.

number of individuals. Both races of paramecium responded in the same manner. The period of depression was practically as marked

from the short exposures as from the longer ones although some incomplete data indicate that long exposures cause an initial stimulation rather than a depression of the division rate.

Experiment 2. To Test the Effect on the Rate of Division of Repeated Exposures to X-Rays.—

(*a*) The exposure was repeated once within 24 hours after the first dose.

Twenty individuals of both races were isolated in separate watch-glasses and for 2 weeks their normal rate of division was recorded. At the end of this period one animal from each line was isolated and allowed to multiply until there were four descendents. Two of these were held as controls and the other two were placed in paper straw tubes and x-rayed for 1 hour. The next day one of the two rayed specimens or its descendents were again exposed for 1 hour. The rate of division was noted each day for 6 days. In all cases, with but one exception, the animals exposed twice behaved as did those that had only one exposure. The rate of division was temporarily lowered but returned to the normal in about 3 days. This experiment has been repeated six times with comparable results with the single exception noted in which the forms died that were exposed twice. In this case, of fourteen paramecia x-rayed on 2 successive days, ten died on the 1st day after the second exposure without dividing, one died without dividing on the 3rd day, one divided and died the 2nd day, one divided and died the 3rd day, and one divided after 3 days and lived. These results have not been again obtained.

(*b*) Exposure repeated twice a day for 8 days.

Mass cultures of both races were exposed in watch-glasses for 10 minutes, at 4 p.m. and at 10 a.m. for 8 days. The animals were exposed fifteen times in all without any obvious reduction in their number as compared with the controls. At the end of the period all appeared slightly swollen and somewhat more sluggish than usual.

(*c*) Three exposures about 12 hours apart.

The technique was similar to that used in (*b*) except that after each exposure ten individuals were isolated from the mass culture and their division rate followed. Two lengths of exposure were used, 15 and 30 minutes. The first two exposures lowered the rate of division below that of the controls although after the second it was not as much lowered as after the first. After the third exposure there was no depression, and, in the case of the 15 minute sample, there was a slight increase in the rate of division. The average number of divisions for the first days after each of the exposures is shown below.

	Time exposed.		
	1	2	3
15 min. exposure	0	0.6	1.4
30 " "	0	0.4	1.2
Control	0.2	0.9	1.2

At the end of 4 days the average division per day for both control and radiated animals was 1.

(*d*) Exposure repeated at intervals of 3 or 4 days.

Since our early work seemed to show that the division rate returned to normal in about 3 days, mass cultures were rayed every 3 or 4 days to determine whether a more enduring effect could be produced. Mass cultures of both races were placed in watch-glasses. One culture of each was held as controls and the other cultures were rayed for 20 minutes. Four individuals from each x-rayed culture were isolated and the rate of division followed for 3 and in some cases 4 days, when the culture was again exposed to x-rays for 20 minutes. This was repeated four times in all and after each exposure four paramecia were isolated from the controls and the x-rayed samples. The C race showed the usual initial depression in the rate of division followed by a complete recovery in 4 days. Each succeeding exposure produced the same effect. The M race had its division rate lowered after the first exposure but after the succeeding exposures it was slightly raised. In all cases, however, the rate of division of the x-rayed samples and of the controls was approximately the same at the end of 4 days.

In the observations described under Experiment 2, we have attempted to extend the initial depression of division by repeating the exposure once or twice daily or at intervals of 3 or 4 days. As many as fifteen exposures have failed to cause a further definite decline in the rate of multiplication or to continue the original depression. One exception to this general statement should be recorded. A number of individual paramecia were rayed for 1 hour and half of them were given a second exposure the following day. During the period that it took the controls to divide five times, the animals receiving a single x-ray dose divided twice and those that had two doses with one exception failed to divide at all and died in from 1 to 3 days. Thus far it has not been possible to duplicate this result.

Repeated doses on mass cultures of paramecium have consistently caused the animals to round out so that they appeared slightly swollen, and to become somewhat sluggish in their movements; but no lethal effect has been noted.

The data presented in Experiment 2 (*c*) and (*d*) seem to indicate that the M race may develop what might be termed an immunity to x-ray after several doses. What actually happens may be that the animal approaches the limit of its reactive power, after which it shows either less or no reaction to this physical agent. In the table of Experiment 2(*c*) the second dose produced less effect than the first,

and the third produced no change in one case and a slight acceleration of division in the other. The same phenomenon is recorded in Experiment 2 (*d*). In this instance the exposures were 3 days apart and after the first dose the division rate was initially stimulated rather than depressed. In both cases, however, the number of animals produced at the end of a given period as in the case of the controls was the same. The length to which this progressive inactivation, if it may so be called, may be carried has not as yet been determined.

To determine whether one end of the cell was more sensitive to x-rays than the other, the anterior and posterior ends of radiated animals were isolated after fission and followed for several generations. No differences in division behavior were noted.

To discover whether exposure to x-rays would be more potent during the process of fission (mitosis) the division of the individual was timed so that it would occur during the process of radiation. The descendents of these animals were studied for several generations but the results were not different from those described above.

DISCUSSION.

The inability of earlier workers to obtain any results after exposing protozoa or small metazoa to x-rays is probably due to the fact that they were largely concerned with attempts to determine a lethal dose. Since the animals did not succumb even to very long exposures it was concluded that they are non-sensitive to this physical agent. To demonstrate the reactions of paramecium to x-rays as reported above it was necessary to observe a large number of individuals and to follow their descendents for several days.

The slight initial check on cell division followed by a rapid recovery is the most obvious of our results. The slight stimulation of the rate of division which occurred after exposures of 5 and 6 hours as well as the results (with Race M) after repeated exposures at intervals of several days suggest that the reaction of paramecium to x-radiation may be reversible. Treatments lasting for 10 minutes to 3 or 4 hours depress division, while longer or repeated exposures may under some conditions raise the reproductive rate.

While it is not possible on the basis of the present data to draw

conclusions regarding the character of the physiological reactions, certain inferences seem warranted. The actual mechanism of reproduction (mitosis) cannot be affected since animals radiated while dividing behaved no differently than the others. It seems more probable that the reaction to x-rays must be a more or less general one throughout the organism, since experiment has shown that one end is no more affected than the other, and since, furthermore, there is apparently a residuum of the primary effect remaining for several days, even after the animal has divided. The residual effect is indicated by the lack of a reaction or the lessened reaction to later exposures at various intervals. This residual effect disappears in 3 or 4 days as far as its influence upon cell division is concerned.

We are able to offer no direct evidence as to why protozoa or small metazoa prove so slightly reactive to doses of x-ray that are many times more than sufficient to kill a small mammal. It may be owing to the circumstance that paramecia very rapidly react to their maximum, after which no change in them occurs.

SUMMARY.

Two races of paramecium were submitted for varying lengths of time to x radiation and a large number of individuals were observed to determine the effect on the rate of division. The division rate of both races suffered a slight initial depression lasting for 2 to 5 days following the exposure. This depression is followed by complete recovery.

Within rather wide limits the length of the exposure has, in these experiments, made no appreciable difference. Apparently the maximum effect of the x-rays is produced by relatively short exposures. Continued radiation produces little further change until exposures of 3 and 4 hours are used, when precisely the opposite results are obtained from those obtained with shorter exposures.

Doses repeated at various intervals have in general failed to interfere more markedly with the division rate than a single dose. Repeated radiation causes the cells to become slightly swollen without apparent interference with their viability.

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STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

V. Effect of Electrolytes on the Rate of Inactivation of Bacteriophage by Alcohol.

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In an earlier paper[1] we have shown that upon the addition of an excess of alcohol to bacteriophage, the activity of the latter is rapidly reduced. This period of rapid inactivation seems roughly to coincide with the process of precipitation occurring when alcohol is added to the lytic filtrate. Further exposure to alcohol affects the residual lytic activity comparatively slowly.[2]

It is known[3–7] that lytic agent is easily adsorbed by various colloids. In our own experience, for instance, mere increase in the concentration of agar in the medium to 1.5 per cent caused loss of over 99 per cent of the activity of lytic filtrate as compared with that observed in the presence of 0.05 per cent of agar.[8,9] It thus seemed possible that the rapid initial inactivation referred to above could be due to the adsorption of lytic agent during precipitation occurring upon the addition of alcohol. In order to see if there exists any relation between the precipitation of the filtrate and the disappearance of its lytic activity, we attempted to vary the intensity of precipitation by controlling the

[1] Bronfenbrenner, J. J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 177.

[2] Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 419.

[3] Doerr, R., *Klin. Woch.*, 1922, i, 1493.

[4] Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 422.

[5] Nakamura, O., *Arch. Hyg.*, 1923–24, xcii, 61.

[6] Brutsaert, P., *Compt. rend. Soc. biol.*, 1924, xc, 1292.

[7] Hauduroy, P., *Compt. rend. Soc. biol.*, 1924, xc, 1463.

[8] Bronfenbrenner, J. J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 315.

[9] Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483.

concentration of electrolytes, and to follow the change in the rate of inactivation of the lytic filtrate caused thereby.

Effect of Excess of Sodium Chloride on the Rate of Inactivation of Bacteriophage by Alcohol.

The general procedure followed in these experiments consisted in mixing, in a series of tubes, 0.5 cc. of active filtrate (prepared in 0.5 per cent NaCl broth pH = 7.4) with equal amounts of sodium chloride solution of varying concentrations. These mixtures were cooled to 7°C.; to each tube except two were added 10 cc. of cooled 95 per cent alcohol; and the whole series was placed in the ice box. Of the two tubes not receiving alcohol, one served as control of the original activity of the filtrate (A), and the other, receiving salt solution of maximum concentration, but no alcohol, was held to indicate the effect of the salt alone (B) on the activity of the filtrate. After 3 hours at 7°C., the contents of the respective tubes were thoroughly mixed and 0.1 cc. samples removed for titration by serial dilution in broth. The contents of Tubes A and B were diluted with 10 cc. of 0.5 per cent NaCl solution, previous to titration, in order to render the concentration of lytic filtrate in them comparable to that in the remaining tubes of the series.

The results of this titration are indicated in Protocol 1 both in terms of the actual amount of the respective mixtures exhibiting lytic activity, and in terms of the approximate amount of the original lytic filtrate present in each. As in the earlier experiments,[2] readings were taken at the end of 24 and 40 hours, and the final readings were checked by means of transfer to broth seeded with susceptible bacteria.

Effect of Precipitation on the Activity of the Alcoholic Solution of the Lytic Agent.

The results of this experiment show that sodium chloride alone does not affect the activity of the lytic agent during 3 hours exposure, even when its concentration in the filtrate reaches 2.59 M (Protocol 1, B). On the other hand, addition of 10 volumes of alcohol in the presence of 0.085 M NaCl reduces the activity of the filtrate about 100 times (Protocol 1, C). As the concentration of sodium chloride in the filtrate increases the precipitation is more complete and inactivation of the lytic principle is more marked; and when the concentration of sodium chloride in the filtrate reaches 1.79 M, its activity is almost completely destroyed (Protocol 1, G and H) during 3 hours incubation

Protocol 1.

Increase in the Rate of Inactivation of Bacteriophage by Alcohol, Due to Addition of NaCl to the Filtrate.

Tube	A	B	C	D	E	F	G	H
	Control.	Control.	Control.					
Lytic filtrate in 0.5 per cent NaCl broth, *cc.*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl solution. Amount, *cc.*	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
NaCl solution. Concentration	0.085 M	5.1 M	0.085 M	0.35 M	0.85 M	1.7 M	3.5 M	5.1 M
Resulting concentration of NaCl (about)	0.085 M	2.59 M	0.085 M	0.21 M	0.46 M	0.89 M	1.79 M	2.59 M
95 per cent alcohol, *cc.*	0	0	10	10	10	10	10	10
Contents of tubes thoroughly mixed and placed at 7°C. for 3 hrs.								
Precipitate	0	0	+	+	+	++	+++	+++
0.5 per cent NaCl solution, *cc.*	10	10	0	0	0	0	0	0

Contents of each tube titrated by serial dilution in broth.

Lytic filtrate present in each dilution (approximate).	Actual amount of mixtures transferred to broth.	A 24 hrs.	A 40 hrs.	A Final transfer.	B 24 hrs.	B 40 hrs.	B Final transfer.	C 24 hrs.	C 40 hrs.	C Final transfer.	D 24 hrs.	D 40 hrs.	D Final transfer.	E 24 hrs.	E 40 hrs.	E Final transfer.	F 24 hrs.	F 40 hrs.	F Final transfer.	G 24 hrs.	G 40 hrs.	G Final transfer.	H 24 hrs.	H 40 hrs.	H Final transfer.
cc.	*cc.*																								
5×10^{-3}	10^{-1}	±	±	+	+	±	+	+	±	+	+	±	+	+	±	+	±	±	+	±	+	+	−	−	−
5×10^{-4}	10^{-2}										+	±	+	+	±	+	+	±	+	±	−	−	−	−	−
5×10^{-5}	10^{-3}	+	±	+	+	±	+	±	+	+	+	±	+	+	±	+	+	+	+	−	−	−	−	−	−
5×10^{-6}	10^{-4}							±	+	+	±	+	+	±	+	+	±	−	−	−	−	−	−	−	−
5×10^{-7}	10^{-5}	±	+	+	±	+	+	±	+	+	±	+	+	±	+	+	±	−	−						
5×10^{-8}	10^{-6}	±	+	+	±	+	+	±	+	+	±	+	+	±	−	−	−	−	−						
5×10^{-9}	10^{-7}	±	±	+	±	+	+	−	−	−	±	−	−	−	−	−	−	−	−						
5×10^{-10}	10^{-8}	±	+	+	±	+	+	−	−	−	−	−	−	−	−	−	−	−	−						
5×10^{-11}	10^{-9}	−	−	−	−	−	−	−	−	−	−	−	−												

The amount of precipitate in the respective tubes was recorded as 0 = no precipitate; + = precipitate present; ++ = moderate precipitate present; +++ = copious precipitate present.

with alcohol at 7°C. The fact that inactivation of the lytic principle in this experiment appears to be of the nature of adsorption secondary to precipitation of the medium, and not analogous to the process of disinfection, as claimed by d'Hérelle, is further suggested by the experiment which follows.

It was seen in earlier experiments[2] that most of the inactivation of lytic agent occurs within a few minutes following the addition of alcohol. Under the conditions of this reaction, apparently not all of the coagulable material is precipitated (Protocol 1 of the present work, C and D), since addition of sodium chloride to the filtrate results in more copious precipitation, with correspondingly greater inactivation of the lytic agent (Protocol 1, F, G, H). It was to be expected, therefore, that if lytic filtrate is mixed with alcohol, and the precipitate is removed, the clear supernatant fluid which is still active[2] will still contain some coagulable material which should come down if sodium chloride is added to it. If inactivation of lytic agent is due to adsorption, this precipitation must be followed by a greater inactivation, as compared with that occurring in the mixture to which no salt was added.

To test the point 1 cc. of cold lytic filtrate (7°C.) was thoroughly mixed with 10 cc. of cold 95 per cent alcohol (7°C.). This mixture was placed on ice, and after 30 minutes the precipitate which was formed was separated by thorough centrifuging at 7°C. (Protocol 2). The supernatant fluid, now containing only a part of its original lytic activity (the greater part being present in the sediment[2]), was placed in two tubes, 5 cc. in each. To one were added 2.5 cc. of cold distilled water (Protocol 2, A), and to the other, 2.5 cc. of cold 5.1 M sodium chloride (Protocol 2, B); both were placed on ice. At intervals, indicated in Protocol 2, portions of each of the mixtures were removed and titrated for lytic activity by serial dilution in broth. The results were read and recorded exactly as in the earlier experiments.[2]

It will be seen from the results recorded in Protocol 2, A that while the activity of the lytic principle in the supernatant fluid (diluted alcohol) remained practically unchanged for hours, inactivation quickly resulted upon addition of sodium chloride to the mixture (Protocol 2, B). The final concentration of sodium chloride did not exceed 1.7 M, and this concentration did not of itself cause inactivation

(Protocol 1, B). However, when the salt solution was added to the clear supernatant fluid, the solution immediately became opalescent, and a fine precipitate soon appeared. Thus it seems that neither the

Protocol 2.

Inactivation of the Alcoholic Solution of Lytic Agent by the Addition of NaCl.

Cold lytic filtrate..... 1 cc.
Cold 95 per cent alcohol..... 10 cc.
Placed on ice and after 30 min. centrifuged at 7°C.*
Supernatant fluid used further as indicated below:

	A	B
Supernatant fluid (7°C.), *cc*.....	5	5
Distilled water (7°C.), *cc*.....	2.5	
5.1 M NaCl (7°C.), *cc*.....		2.5

Placed on ice and samples removed for titration at intervals.

Time of removal of samples, *hrs*.....	1			3			8			1			3			8		
Amount of precipitate observed.....	−			−			−			+			+			+		
Absolute amount of filtrate present in each tube.	Actual amounts transferred to broth.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.	24 hrs.	40 hrs.	Final transfer.
cc.	*cc.*																		
7×10^{-3}	10^{-1}	+	±	+	+	−	+	+	+	+	±	−	−	−	−	−	−	−	−
7×10^{-4}	10^{-2}	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
7×10^{-5}	10^{-3}	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
7×10^{-6}	10^{-4}	+	+	+	+	+	+	±	+	+	−	−	−	−	−	−	−	−	−
7×10^{-7}	10^{-5}	+	+	+	±	+	+	±	+	+	−	−	−	−	−	−	−	−	−
7×10^{-8}	10^{-6}	±	−	−	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7×10^{-9}	10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7×10^{-10}	10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

* It is important to keep the temperature as low as possible during centrifuging to avoid an excessive destruction of lytic principle (compare Tables II and III in the second paper of the series[2]).

alcohol nor the salt alone causes inactivation of lytic agent; but when the solution of lytic agent in alcohol is caused to precipitate by the addition of salt, a rapid inactivation of lytic agent is the result.

Effect of Valency of Electrolytes on the Rate of Inactivation of Lytic Agent in the Presence of Alcohol.

If the observed increase in the rate of inactivation of lytic agent by alcohol in the presence of sodium chloride is due, as suggested, to a more complete precipitation of the medium, replacement of sodium chloride by salts with polyvalent ions might be expected to increase further the rate of precipitation and thus to result in a more marked change in the same direction.[10] Or, the same degree of inactivation as that produced with a given concentration of NaCl should be brought about by correspondingly lower concentrations of salts with polyvalent ions.

Since precipitation of colloids may be affected by both anions and cations, depending on their charge,[11,12] we attempted in a preliminary experiment to establish which of the ions of a salt are most active under the conditions of our experiment. Accordingly, we compared the effect of an arbitrarily chosen equimolar solution of three salts: sodium chloride, sodium sulfate, and calcium chloride. The procedure was exactly the same as that illustrated in Protocol 1, except that only one concentration of each salt (0.2 M) was used instead of different concentrations of one salt as before (Protocol 3).

Since sodium chloride and sodium sulfate behaved in the same way, it appears that the valency of the anion was of no significance. On the other hand, the calcium salt showed a considerably greater effect on inactivation than did sodium chloride, although the anion in both cases was the same. It was thus obvious that, in the case of the substances studied at least, the nature of the cation was important in this reaction. In the subsequent study of the effect of valency of ions on the rate of inactivation of the lytic principle, the valency of the cation only was considered.

In the preceding experiments, the duration of exposure of the lytic agent to the combined action of alcohol and salt, before titration, was arbitrarily set at 3 hours. Since one salt might be expected to act more quickly than another, in the following experiment samples of the mixtures were removed from time to time, as indicated in the protocol.

[10] Schulze, H., *J. prakt. Chem.*, 1881–82, xxv, N.S., 431; 1884, xxvii, 320.
[11] Hardy, W. B., *Z. physik. Chem.*, 1900, xxxiii, 385.
[12] Pauli, W., and Flecker, L., *Biochem. Z.*, 1912, xli, 461.

Thus we obtained not only an idea of the total effect after 3 hours, but also curves representing the rates of inactivation of the lytic agent due to each salt. In order to avoid unnecessary repetition, only one such experiment (with $MnCl_2$) will be described in detail. It is understood that in the case of each salt exactly the same procedure was followed throughout, with variation only in the concentration of salts until such concentrations were found for each as caused an inactivation of the lytic filtrate comparable with that produced by alcohol in the presence of 1.7 M sodium chloride.

Protocol 3.

Comparative Effect of Cations and Anions on Inactivation of Bacteriophage by Alcohol.

	A	B	C	D
	Control.	NaCl	Na_2SO_4	$CaCl_2$
Lytic filtrate, *cc.*	0.5	0.5	0.5	0.5
0.4 M solution of salts, *cc.*		0.5	0.5	0.5
Distilled water, *cc.*	0.5			
Resulting concentration of salts	0.04 M NaCl	0.24 M NaCl	(0.04 M NaCl) 0.2 M Na_2SO_4	(0.04 M NaCl) 0.2 M $CaCl_2$
Mixtures were cooled to 7°C.				
Cold 95 per cent alcohol, *cc.*	10	10	10	10
Placed on ice for 3 hrs. and titrated.				

Absolute amount of lytic agent present in each tube.	Actual amounts transferred to broth.	A 24 hrs.	A 40 hrs.	A Final transfer.	B 24 hrs.	B 40 hrs.	B Final transfer.	C 24 hrs.	C 40 hrs.	C Final transfer.	D 24 hrs.	D 40 hrs.	D Final transfer.
cc.	*cc.*												
5×10^{-3}	10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4}	10^{-2}	+	+	+	+	+	+	±	+	+	±	+	+
5×10^{-5}	10^{-3}	+	±	+	+	±	+	±	+	+	±	+	+
5×10^{-6}	10^{-4}	±	+	+	±	+	+	±	+	+	−	−	−
5×10^{-7}	10^{-5}	±	+	+	±	+	+	−	+	+	−	−	−
5×10^{-8}	10^{-6}	±	+	+	−	−	−	−	−	−	−	−	−
5×10^{-9}	10^{-7}	−	−	−	−	−	−	−	−	−	−	−	−
5×10^{-10}	10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−

Protocol 4.

Comparative Effect of Different Concentrations of $MnCl_2$ on the Rate of Inactivation of the Lytic Agent in the Presence of Alcohol.

Salt used	NaCl					$MnCl_2$														
Laudman Shiga filtrate, *cc.*	1					1					1					1				
Concentration of salt used	3.4 M					1 M					0.3 M					0.1 M				
Amount of salt solution used, *cc.*	1					1					1					1				
Approximate resulting concentration of salt	1.7 M					0.5 M					0.15 M					0.05 M				
Amount of the above mixture (A), *cc.*	1				1 (control).	1				1 (control).	1				1 (control).	1				1 (control).
Cold water, *cc.*					10					10					10					10
Cold alcohol, *cc.*	10					10					10					10				
Duration of exposure at 7°C.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.	5 min.	15 min.	1 hr.	3 hrs.	3 hrs.
Amount of original filtrate in each tube. / Amount of mixtures used.																				
cc. / *cc.*																				
5×10^{-3} / 1×10^{-1}	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4} / 1×10^{-2}	+	+	−	−	+	+	−	−	−	+	+	+	−	−	+	+	+	+	+	+
5×10^{-5} / 1×10^{-3}	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	+	+
5×10^{-6} / 1×10^{-4}	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	−	+
5×10^{-7} / 1×10^{-5}	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	+	+	+	−	+
5×10^{-8} / 1×10^{-6}	+	−	−	−	+	−	−	−	−	+	−	−	−	−	+	+	+	−	−	+
5×10^{-9} / 1×10^{-7}	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	+	−	−	−	+
5×10^{-10} / 1×10^{-8}	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+	−	−	−	−	+
5×10^{-11} / 1×10^{-9}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

In each case, to several 1 cc. portions of Laudman Shiga lytic filtrate (prepared in unbuffered broth), was added 1 cc. of varying concentrations of salts (in this case $MnCl_2$); after thorough mixing and cooling to 7°C., two portions of 1 cc. each of this Mixture A were removed. To one of these were added, in each case, 10 cc. of cold alcohol, to the other 10 cc. of cold water, and both sets of tubes were placed in the ice box. At intervals indicated in Protocol 4, B, the contents of the tubes containing alcohol were thoroughly mixed, after which samples were removed for immediate titration by serial dilution in broth, and the tubes were replaced in the ice box. After the last titration (3 hours after the beginning of the experiment), the second tube of each series which received no alcohol was also titrated to show what effect, if any, the salt alone had on the activity of the filtrate during the entire period of the experiment. The results of

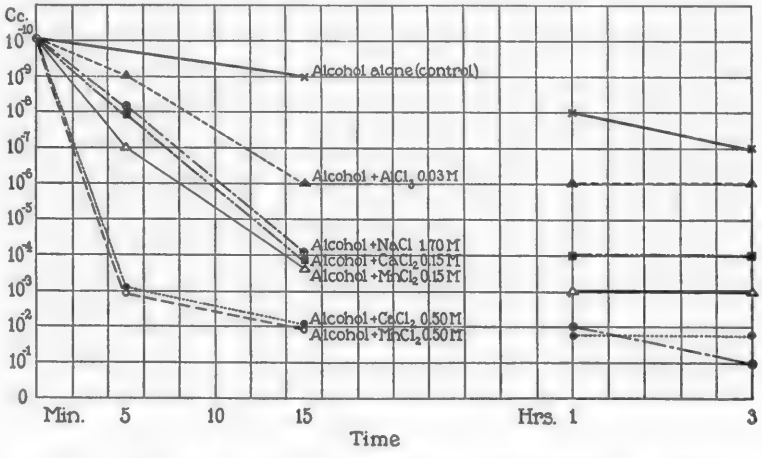

CHART 1. Effect of electrolytes on inactivation of the lytic filtrate by alcohol.

these titrations were read exactly as before (Protocol 1), that is after 24 hours, 40 hours, and after a subsequent control transfer to fresh broth,—but for the sake of simplicity only the final reading has been recorded in Protocol 4.

As can be seen from Protocol 4, the rate of inactivation of lytic agent in the presence of 1.7 M NaCl was most closely approached in the tube containing 0.15 M $MnCl_2$. On the composite Chart 1 are plotted the results of several such experiments. This chart shows again that immediately after the addition of alcohol there occurred a rapid diminution in the activity of the filtrate. This inactivation proceeded

at a fairly constant rate for about 15 minutes. Later, the curves show a distinct tendency to flatten, possibly coincident with the completion of precipitation. This chart shows further that approximately the same rate of inactivation resulted in the presence of 1.7 M sodium chloride as in that of 0.15 M calcium chloride or 0.15 M manganese chloride. In the case of aluminum chloride or lanthanum chloride, a concentration of a little over 0.03 M would accomplish approximately the same inactivation.[13]

Thus, in general, salts with polyvalent cations increase the rate of inactivation of lytic filtrate in the presence of alcohol much more than does sodium chloride under similar conditions. The greater the valency of the cation, the greater is the rate of inactivation. Although there were some minor deviations, which will be discussed in detail in one of the later papers of this series, the nature of cation as apart from its valency influenced the respective rates of inactivation of the lytic agent but little; in many instances no measurable difference could be detected. Thus, for instance, the increase in the rate of inactivation of the lytic agent resulting from the increase in the concentration of calcium chloride from 0.15 M to 0.5 M was duplicated by a corresponding change in the concentration of manganese chloride (Chart 1). Similar parallelism was observed when the concentrations of $AlCl_3$ and $LaCl_3$ were varied.

Effect of Partial Removal of Salts by Dialysis on the Rate of Inactivation of Bacteriophage by Alcohol.

The results of the preceding experiments consistently indicated an intimate relation between the rate of precipitation of lytic filtrate and the loss of its activity resulting from the addition of alcohol. It has been shown that addition of electrolytes increases this inactivation, and that the rate of increase depends on the valency of the cation. Since the lytic filtrate, consisting of beef infusion broth, already contains various salts in addition to 0.5 per cent sodium chloride added to it in the process of preparation, it was thought that perhaps even the initial inactivation of bacteriophage by alcohol[2] (prior to the addition

[13] The two latter salts could not have been used in concentrations higher than 0.03 M on account of the fact that the change in hydrogen ion concentration in the solution attained thereby was detrimental to the activity of the lytic agent.

Protocol 5.

Activity of Dialyzed Lytic Filtrate.

Actual amount of fluid having lytic activity.	A	B										C		D
	Original lytic filtrate.	Outer fluid after first 24 hrs. of dialysis.		Outer fluid after second 24 hrs. of dialysis.		Outer fluid after third 24 hrs. of dialysis.		Outer fluid after fourth 24 hrs. of dialysis.		Outer fluid after fifth 24 hrs. of dialysis.		Dialyzed lytic filtrate (after 5 days of dialysis).		Original lytic filtrate kept on ice for 7 days.
		Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.	Chloride.	Lytic power.
cc.														
1×10^{-1}	+	+	Present.	+	Present.	+	Present.	+	Present.	+	Tr.	+	Present.	+
1×10^{-2}	+	+		+		+		+		+		+		+
1×10^{-3}	+	+		+		+		+		+		+		+
1×10^{-4}	+	+		+		+		+		+		+		+
1×10^{-5}	+	+		+		+		+		+		+		+
1×10^{-6}	+	+		+		+		−		+		+		+
1×10^{-7}	+	−		−		−		−		−		+		+
1×10^{-8}	+	−		−		−		−		−		+		+
1×10^{-9}	+	−		−		−		−		−		+		+
1×10^{-10}	−	−		−		−		−		−		−		−
1×10^{-11}	−	−		−		−		−		−		−		−

of electrolytes) might be due largely to this presence of salts in it. We therefore attempted to reduce the salt content of the lytic filtrate by dialysis, and to compare the rate of inactivation (by alcohol) of the resulting lytic filtrate with that of the original filtrate.

For this purpose, 50 cc. of ordinary beef infusion broth containing 1 per cent of peptone and 0.5 per cent of sodium chloride, and adjusted to neutral reaction, were seeded with 0.1 cc. of a suspension of young *B. dysenteriæ* Shiga (grown for 18 hours on an agar slant) containing 100.000,000 microorganisms, and incubated at 37°C. for 1 hour. At this time, 0.1 cc. of a dilution of Laudman Shiga lytic principle 1:10,000 in broth was added to the culture. The latter was further incubated for 40 hours, filtered, and the filtrate placed on ice after having been tested for sterility and for its lytic action (Protocol 5, A). 2 days later, when the tests showed the filtrate to be sterile, a 5 cc. portion of it was removed and placed in a sterile collodion thimble surrounded by 200 cc. of sterile distilled water. Both the dialyzing thimble and the remaining filtrate were replaced on ice. The next day, as well as on each succeeding one, the liquid surrounding the dialyzing thimble was removed, tested for the presence of chlorides, and titrated for its lytic activity (Protocol 5, B). At the same time, 200 cc. of fresh sterile distilled water were introduced in place of the liquid removed, and the flask was returned to the ice chest. After 5 days of dialysis against distilled water, the contents of the collodion bag were likewise subjected to the qualitative test for chlorides, and to titration for their lytic power (Protocol 5, C) as well as for sterility. At the same time, a portion of the original filtrate was also titrated for comparison (Protocol 5, D). It will be observed that the lytic filtrate remained sterile; that it was not entirely freed of chlorides by the end of 5 days of dialysis (Protocol 5, C); also, together with chlorides, a certain portion of lytic agent dialyzed and thus was removed daily (Protocol 5, B). This amount was so small, however, that it was not detected when the contents of the bag were titrated after the 5 days of dialysis (compare C and D in Protocol 5) by the usual method of tenfold dilution.

Having thus established the fact that lytic filtrate, partially dialyzed against distilled water, retained practically its full activity in so far as the method of titration permitted estimation, we next proceeded to inquire into the effect of alcohol upon it.

Two portions of 3 cc. each of dialyzed lytic filtrate were placed in tubes;[14] to one of them (Protocol 6, A) was added 0.05 cc. of water, and to the other 0.05 cc. of 30 per cent sodium chloride solution (Protocol 6, B). At the same time, a 5 cc.

[14] The filtrate, during dialysis, became diluted through osmosis, so that instead of the original volume of liquid placed in the bag (5 cc.), the bag contained by the end of the 5th day approximately 11 cc. of fluid.

Protocol 6.

Effect of Partial Dialysis on Resistance of Bacteriophage to Alcohol.

	A	B	C
Dialyzed filtrate (diluted 5:11 during dialysis), *cc.*	3	3	
Original filtrate (diluted 5:11), *cc.*			3
0.5 per cent NaCl solution, *cc.*			0.05
Distilled water, *cc.*	0.05		
30 per cent NaCl solution, *cc.*		0.05	
Resulting concentration of NaCl	Definite traces.	About 0.5 per cent.	About 0.5 per cent.
Placed on ice for 3 hrs.			
From the above mixtures (cold), *cc.*	1	1	1
95 per cent alcohol (cold), *cc.*	10	10	10

Placed on ice and titrated at intervals.

Duration of exposure to alcohol		A 15 min.	A 1 hr.	A 3 hrs.	A 24 hrs.	A 48 hrs.	B 15 min.	B 1 hr.	B 3 hrs.	B 24 hrs.	B 48 hrs.	C 15 min.	C 1 hr.	C 3 hrs.	C 24 hrs.	C 48 hrs.
Amount of original filtrate in each tube.	Amount of mixtures used.															
cc.	*cc.*															
5×10^{-2}	1×10^{-0}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-3}	1×10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-4}	1×10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5×10^{-5}	1×10^{-3}	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
5×10^{-6}	1×10^{-4}	+	+	+	+	+	+	+	+	+	−	+	+	+	−	−
5×10^{-7}	1×10^{-5}	+	+	+	+	+	+	+	+	−	−	+	+	+	−	−
5×10^{-8}	1×10^{-6}	+	+	+	+	−	+	−	−	−	−	+	+	−	−	−
5×10^{-9}	1×10^{-7}	+	+	+	−	−	+	−	−	−	−	+	−	−	−	−
5×10^{-10}	1×10^{-8}	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

portion of the original filtrate (not subjected to dialysis) was diluted to 11 cc. with a 0.5 per cent NaCl solution (in order to duplicate the dilution of the dialyzed portion), 3 cc. of the resulting mixture were placed in a third tube (Protocol 6, C), and to this was further added 0.05 cc. of 0.5 per cent NaCl solution. The contents of the tubes were thoroughly mixed, and now 1 cc. portions of each were put into cooled tubes and placed on ice. To the contents of each tube, after cooling, were added 10 cc. of cold 95 per cent alcohol, the tubes were placed on ice, and after thorough mixing of the contents samples were titrated at intervals, as indicated in Protocol 6.

The findings of this experiment indicate that removal of a part of the salts from the lytic filtrate resulted in its greater resistance to alcohol (Protocol 6, A), and that replacement of sodium chloride in the dialysate to approximately the same concentration as that in the original broth results in the increase in its sensitiveness to alcohol (Protocol 6, B). That in the latter case (Protocol 6, B) the rate of destruction does not quite attain the level observed in the control (Protocol 6, C) is possibly due to the fact that, during dialysis, in addition to sodium chloride some other salts were also removed from the filtrate.

DISCUSSION.

The results of the experiments reported in this paper suggest, in our opinion, that the inactivation of the lytic principle by alcohol is not due to the virucidal action of the latter, but to the adsorption of dissolved lytic substance on the surface of the precipitate occurring when alcohol is added to the lytic filtrate. However, our results lack the character of indisputable proof. Thus, for instance, those in favor of the parasitic nature of bacteriophage might take the view that the addition of electrolytes in our experiments increased the disinfecting action of alcohol by forcing it out of the water, and concentrating it in the lipoid constituents of the supposed cell membrane of the "*bacteriophagum intestinale.*"

We shall consider this possibility in later experiments. For the present, however, such an explanation does not seem satisfactory. In the first place, such analogy with the effect of sodium chloride on the bactericidal effect of phenol[15] does not suffice to explain the effect of valency of salts as observed in our experiments. Furthermore, if the

[15] Spiro, K., and Bruns, H., *Arch. exp. Path. u. Pharmakol.*, 1898, xli, 355.

inactivation of lytic agent were due in the final analysis to direct toxic action of alcohol, one would expect the rate of the reaction to be expressed by a straight line, as is the case with the rate of lysis of erythrocytes by alkalies,[16,17] for example, or with the rate of destruction of bacterial spores by mercuric chloride,[18] as calculated by Morawitz.[19] As a matter of fact, we have seen that for the first few minutes the inactivation of lytic agent by alcohol proceeds at a rate consistent with the formula proposed by Ostwald for disinfection, but soon the curves flatten out, in spite of the presence of an excess of both bacteriophage and alcohol in the solution. This break in the rate of inactivation of the lytic agent suggests that the reaction which takes place is not due to the alcohol directly, but to some other factor set into action by the addition of alcohol, but ceasing to operate after the first few minutes. We believe this factor is the formation of a fine precipitate with the concomitant adsorption of the lytic agent.

The adherents of the vitalistic theory of the bacteriophage action might suggest that the filtrate representing, according to their views, a suspension of "*bacteriophagum intestinale*," by analogy with bacteria, might be composed of variants possessing varying degrees of susceptibility to the destructive effects of alcohol. Thus, if the majority of individuals were highly susceptible, and the remainder highly resistant to toxic action, the result would be, in general, similar to that which we observed; namely, the majority of the hypothetical organisms would be destroyed soon after the addition of alcohol, and the filtrate would show a marked and rapid fall in its activity. The remainder of the organisms would be destroyed at a different and much slower rate and consequently the curve expressing the rate of inactivation would show a break.

That such a view is untenable follows from our earlier findings that lytic filtrates are uniform in their susceptibility to the action of alcohol, and contain no fraction possessing a higher degree of resistance.[2]

Still another objection might be raised against our interpretation of

[16] Gros, O., *Biochem. Z.*, 1910, xxix, 350.
[17] Stadler, E., and Kleemann, H., *Biochem. Z.*, 1911, xxxvi, 301, 321.
[18] Krönig, B., and Paul, T., *Z. Hyg. u. Infectionskrankh.*, 1897, xxv, 1.
[19] Morawitz, H., *Kolloidchem. Beihefte*, 1909–10, i, 301.

the results here presented. Lytic agents have been adsorbed on a number of organic and inorganic colloids. Usually they are not permanently inactivated thereby. In the case of the adsorption of the lytic agent by the precipitate resulting from precipitation of the lytic filtrate by alcohol, however, there occurs a marked irreversible reduction in its activity. This destruction, in our opinion, finds its analogy in the similar effect of alcohol upon enzymes and toxins, and is due to denaturation of the protein vehicle on which lytic agent is distributed in a very thin (monomolecular ?) layer.

Somewhat similar observations may be quoted with respect to the destruction of bacteriophage by heat. It has been found by a number of investigators that bacteriophage is relatively heat-resistant, and requires at least ½ hour's exposure at 70–90°C. for complete destruction. However, when it is adsorbed on the surface of bacteria, it is destroyed at 56°C.[20]

Another analogy is seen in our experiments[21] in which bacteriophage is completely inactivated by acetone if electrolytes are added to the filtrate before precipitation, whereas without the addition of electrolytes bacteriophage is found practically unaffected by the acetone.

SUMMARY AND CONCLUSIONS.

Addition of neutral salts to the lytic filtrate results in an increased rate of inactivation of the latter when alcohol is added to it. This effect of salts is the more marked the higher the valency of the cation. Conversely, removal by dialysis of salts originally present in the lytic filtrate tends to render lytic agent less sensitive to alcohol. Restitution of the original salt content to the dialyzed filtrate tends to bring the sensitiveness to alcohol in the dialyzed filtrate to the level of the non-dialyzed control.

It appears, therefore, that inactivation of the lytic agent by alcohol depends directly on the rate of precipitation of the coagulable constituents of the medium, and is not the result of a direct toxic action of alcohol on "*bacteriophagum intestinale*." Considered in association with our earlier findings, these results speak in favor of the chemical nature of the agent of transmissible lysis.

[20] Seiffert, W., *Med. Klin.*, 1922, xviii, 997, 1093, 1121.

[21] Bronfenbrenner, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1925 (in press).

THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

I. General Considerations of the Reaction.

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During the course of studies on oxidation and reduction by pneumococcus made by Avery and Neill (1), Neill made the observation that when pneumococcus extract was injected into white mice hemorrhagic purpura developed. At a later date, we made a similar observation. This paper is a report of the study of the nature of this phenomenon.

In 1907, Heyrovsky (2) reported that he was able to produce purpura in white mice by injecting 24 hour filtrates of virulent pneumococci grown in dextrose broth. No other reference to this reaction has come to our attention.

We have found that if pneumococcus extracts, prepared by one of the various methods to be described later, are injected into white mice, after 4 to 6 hours the skin over the feet, tail, ears, snout, and genitalia take on a dark bluish purple color. This peculiar appearance is confined to the areas where the hair is either absent or scanty, but removal of the hair does not render the skin in the shaved area reactive. The intensity of the discoloration varies in the different regions and only one or several or all of the sites mentioned may be involved. Unless the amount of extract injected is very large the animals show no signs of intoxication and all recover. The lesion is at its maximum intensity usually in 24 to 48 hours following the injection and then slowly disappears, vanishing entirely in 5 to 7 days.

The development of the reaction may best be observed in the ear by transmitted light. It is then seen that the lesion begins as a small hemorrhage about the blood vessels and the extravasation extends into the surrounding tissues.

The microscopical changes[1] are best studied in sections made of the skin and viscera of animals autopsied immediately after they have died or been killed by chloroform vapor. It is then found that the changes are not confined to the skin but are also present in the lungs and voluntary muscles. The essential lesion consists of extravasations of blood into the loose tissues surrounding the blood vessels. In the skin the hemorrhages are usually found in the subcutaneous tissues, but may also be present in the superficial layers of the corium. The hemorrhages may be minute or may involve quite extensive areas. Frequently small collections of polymorphonuclear leucocytes are present in the hemorrhagic areas.

The degree of purpura is influenced both by the amount of the material introduced and the individual susceptibility of the experimental animal. Using a fixed quantity of extract for a series of mice there is considerable variation in the severity of the reaction in the individual animals. 0.2 cc. of extract usually produces purpura in half of the mice of a series. 0.4 cc. gives 90 per cent or more positive results with rather severe reactions. More than this amount proves too toxic and death usually occurs, although some mice have been able to withstand 0.6 cc. and, rarely, 0.8 cc. of the extract.

The reaction is produced equally well whether the extract is introduced subcutaneously, intraperitoneally, or intravenously. The intravenous method usually results in a more rapid reaction. Attempts to effect purpura by feeding have not, however, been successful. After being subjected to chloroform, mice were fed by stomach tube with amounts much greater than necessary to cause purpura if injected, but in these cases no purpura was evidenced. This is not surprising since trypsin destroys the activity of the purpura-producing principle.

Purpura was produced in guinea pigs and rabbits also. In the guinea pig the ears and the pads of the feet were affected; while the ears and scrotum of male rabbits,—which alone were used,—were chiefly involved.

Derivatives of Pneumococcus Causing Purpura.

In our study the development of purpura was first observed in white mice as a response to injections of pneumococcus extract pre-

[1] The pathology of the effect of pneumococcus extract in experimental animals is under study by Dr. Branch and a report upon it will be made subsequently.

pared according to the method of Avery and Neill (3). Purpura was also produced in white mice by Heyrovsky's method. Cultures of pneumococcus were grown in dextrose broth and a 1 day's growth was filtered and injected intraperitoneally. The reaction, however, was much less constant and less severe than that obtained by the introduction of extract. Moreover, when purpura did appear, as much as 1.5 to 2.0 cc. were required to produce the reaction. Filtrates of young cultures (8 to 16 hours) grown in plain beef infusion broth, however, have never yielded purpuric lesions. Filtrates from very old cultures grown in plain infusion broth for 3 weeks only rarely have given purpuric reactions and when they did the activity was slight.

Whole cultures, unfiltered, rarely cause the purpuric reaction. Young cultures usually cause an infection followed by rapid death of the mice. In hundreds of mice injected with young cultures of pneumococcus for one purpose or another never but once has purpura followed the injection. On this occasion the mouse had been injected with a virulent culture together with sufficient immune serum to afford passive protection. On the 5th day following the injection purpura was noted. It increased in intensity until the 7th day and then began to blanch. The infection itself, however, does not prevent the appearance of purpura, for purpura results when an active extract is injected into an animal together with a minimum infecting dose of culture.

Old cultures occasionally produce purpura. Two strains of Type III have given the most consistent results. After the original 24 hour culture in blood broth had been stored for a week or more in the ice chest, these strains were able to produce purpuric changes. Since the mice were not severely infected, it is probable that during storage the cultures had undergone sufficient autolysis to be partially transformed into extract-like substances.

Bile-dissolved cultures have never induced purpura, even when solutions prepared from young cultures of pneumococci, concentrated to the same extent as is done in preparing extracts, and dissolved in a minimum quantity of bile, are employed. That bile in itself does not inhibit the purpurogenic activity of an extract was shown by adding bile to an active extract in a concentration of 10 per cent. This mixture injected into mice produced purpura.

Pneumococci which had been killed by exposure to a temperature of 56°C. for 30 minutes,—a treatment sufficient to kill the pneumococci but not to destroy the activity of the purpura-producing material,—did not produce purpura in white mice. The soluble specific substance and nucleoprotein of pneumococcus (Heidelberger and Avery (4)) failed to produce purpura regardless of the quantity injected.

The observations so far recorded suggest that the substance, which on injection gives rise to purpura, is contained in the bodies of the pneumococci and is set free by the process of extraction employed. It is also found occasionally in the culture filtrate. It has been of importance to determine whether this substance exists preformed in the bacterial cell or whether it is a product of a digestive process occurring during the process of extraction.

Concentrated suspensions of pneumococci were prepared by centrifuging 1500 cc. of a 9 hour broth culture and suspending the sediment in 15 cc. of broth. When trypsin was allowed to act on a portion of this concentrated suspension the resulting fluid was not active. This was the case whether the bacteria in the emulsion were alive or had been killed by heat before exposure to the action of trypsin.

If, instead of trypsin, the native enzymes of the pneumococcus were allowed to act on the concentrated emulsion, the results were quite different, since the product in this case was highly active. In this experiment to 15 cc. of the concentrated pneumococcus emulsion, 10 cc. of an homologous extract containing the pneumococcus enzymes (Avery and Cullen (5)) were added and the mixture was incubated for 40 hours at 37°C. The mixture was then centrifuged and the supernatant fluid filtered. This filtrate was now found to be as active as the pneumococcus extract prepared by the Avery-Neill method.

The Nature of the Purpura-Producing Principle.

Aliquot portions of pneumococcus extract were heated at 60°C., 70°C., 80°C., and 100°C. for 10 minutes each. At the higher temperature exposures, coagulation occurred. The various specimens were injected individually into the peritoneum of white mice and it was observed that heating even at 100°C. for 10 minutes did not destroy the activity of the purpura-producing material. The results are appended in Table I.

Avery and Neill were able to destroy both the hemolytic and oxidation-reduction activity of extracts by exposure to air. It was of

interest to determine, therefore, the effect of oxidation on the purpura-producing principle of the extracts. Pneumococcus extract was oxidized until it was no longer hemolytic. It was still capable of producing purpura when injected into white mice. It can be said, then, that the purpura-producing principle is not associated with the hemolytic activity of the extracts, since the latter alone is destroyed by heat and oxidation.

Extracts of all groups of pneumococci contain the purpura-producing material. Experiments performed to correlate the purpura-producing activity with virulence showed no relationship between these two properties. An avirulent Group IV strain gave as good a purpura-producing extract as any of the most highly virulent strains in this laboratory.

TABLE I.

The Effect of Heat on the Purpura-Producing Material.

Each mouse was injected intraperitoneally wth 0.4 cc. of the respective extract.

Type of extract.	No. of mice in test.	No. of mice developing purpura.
Unheated extract	4	3
Extract heated 10 min. at 60°C.	2	2
" " 10 " " 70° "	2	2
" " 10 " " 80° "	2	1
" " 10 " " 100° "	6	5

Because it has been shown (6, 7) that an avirulent culture of pneumococcus may be a strain composed of both virulent and avirulent organisms, the experiment was extended to include a study of the extracts prepared from the R or purely avirulent strains. It was shown that the extracts possessed purpura-producing material.

Isolation of the Purpura-Producing Material.

For the isolation of the purpura-producing material from the extracts, saline extracts of pneumococcus were prepared according to the technique of Avery and Neill. These extracts were equivalent to the broth extracts in producing purpura. Normal acetic acid was added slowly to the saline extract until no further precipitation occurred. The material was centrifuged and all of the supernatant was withdrawn. The sediment was washed with normal salt solution, then dissolved in weak alkali and neutralized, and the volume was adjusted to the original

quantity. The supernatant no longer gave a precipitate with acetic acid. It was neutralized with normal NaOH.

The supernatant was next half saturated with $(NH_4)_2SO_4$ and a precipitate was obtained. This was centrifuged and the supernatant was collected and made up to full saturation with $(NH_4)_2SO_4$ and another precipitation was obtained.

The acetic acid precipitate, which contained essentially the nucleoproteins of pneumococcus (4) produced no purpuric lesions when injected into white mice. This was in accordance with the earlier observation on the nucleoprotein fraction. The supernatant from the acetic acid precipitation, on the other hand, was still able to produce purpura with apparently no loss in this property. The precipitate obtained by half saturation with $(NH_4)_2SO_4$ did not contain the purpura material while the supernatant was still purpura-producing. The precipitate obtained by full saturation with $(NH_4)_2SO_4$ was shown to contain the purpura-producing principle. This precipitate was washed free of salts by dialysis.

It was found that the purified purpura-producing principle was coagulated on heating and was precipitated by trichloracetic acid, sulfosalicylic acid, and alcohol. It gave a positive biuret reaction, but did not give a xanthoproteic reaction and the Millon reaction was a doubtful one. The activity to cause purpura was destroyed by trypsin digestion.

The purified material acted like the extracts themselves and mice showed the same degree of variation in susceptibility to it that they did to the extracts. As small an amount as 0.04 mg.,[2]—a dosage of about 1/500,000 of the body weight,—was sufficient to produce purpura in some of the mice. It proved to be highly toxic for rabbits, and 0.16 to 3.2 mg. was fatal to 3 rabbits. The purpura-producing substance was not precipitated either by anti-extract or antipneumococcus sera.

Purpuric Responses to Extracts from Other Bacteria.

Experiments were conducted to determine whether the purpura-producing material was peculiar to pneumococcus alone. Extracts

[2] These quantities were calculated as protein on the basis of the organic nitrogen in the solution obtained by full saturation of the extract with $MgSO_4$, after precipitation with acetic acid.

were made of two strains of *Staphylococcus aureus*, three strains of *Streptococcus viridans*, one strain of hemolytic streptococcus, and one strain of *Bacillus coli*.

For these organisms[3] the preparation of the extracts was modified, since rapid freezing and thawing did not give solutions of the bacterial cells. The cultures were centrifuged and to the sediment was added a small amount of sterile sand. The bacteria were then dried *in vacuo* at a temperature not exceeding 37°C. and then ground in a ball mill at room temperature for varying periods of 48 hours or more until a fine powder was obtained. At this stage N/100 alkali was added and extraction was allowed to take place in the ice chest, in the ball mill. On the following day the material was removed and centrifuged and the supernatant was used in the experiments. Care was taken to keep the concentrations equivalent to that of the pneumococcus extract.

Extracts were prepared from four strains of meningococcus by freezing and thawing.

The extracts from these organisms were not capable of producing purpura in white mice. Amounts as great as 2.5 cc. were used for injection.

DISCUSSION.

Evidence has already been presented to show that pneumococcus extracts possess a powerful hemolysin and an oxidation-reduction system which is operative on hemoglobin (Avery and Neill). The present paper offers data to show that the extracts possess in addition a toxic principle which is capable of producing experimental purpura in animals. This activity is derived from extracts of avirulent as well as of virulent pneumococci. Extracts of the other bacteria tested either do not possess this activity, or the methods which were applicable for pneumococcus were inadequate to demonstrate it.

The purpura-producing material is obtained from the extracts by full saturation with $(NH_4)_2SO_4$. Whether it is an albumin or some closely related substance as a higher proteose, for example, for which albumin may act merely as a vehicle remains to be determined.

It is significant that bile solutions of pneumococci did not yield the purpura-producing substance. That, on the other hand, bile does not prevent its activity has also been shown. And those constitu-

[3] We are indebted to R. C. Lancefield for this method.

ents of the pneumococcus which exist preformed in the cell are recoverable by dissolution of the cell with bile. The method has been used successfully by Cole (8) to obtain the hemolysin, and by Avery and Cullen (9) to obtain the enzymes of pneumococcus. The purpuric substance was found to be present in those materials alone which represented degradation products of pneumococci. It appears probable, therefore, that the purpura-producing property is due not to a constituent of the living pneumococcus cell, but to a degradation product of pneumococcus. That it is a separate entity from the pneumococcus hemotoxin has been shown selectively. The hemotoxin can be eliminated by oxidation and heat, while neither of these means destroys the activity of the purpura-producing material. Autolysates of pneumococcus are definitely purpura-producing, yet, as prepared by our method, they contain no demonstrable hemotoxin. The fact that autolysates were purpura-producing while autolyzed cultures were rarely, and at best, poorly purpura-producing, is explainable on the basis of concentration, since the autolysates were 50 times more concentrated than the cultures.

It is well recognized that purpura accompanying pneumococcus infections in man is of extreme rarity. There are, however, occasional instances recorded in the literature. Bazan (10) and Nobécourt and Mathieu (11) have reported upon them. In their cases the purpura always preceded death. Whether there is any relation between the purpura-producing principle and the pneumococcus infections cannot be said at the present time. It is quite possible that the purpura substance may function in infections without necessarily producing visible signs of purpura. Experiments are planned to determine whether the purpura principle plays any part in pneumococcus infections.

CONCLUSIONS.

1. Pneumococcus extracts have been shown to be capable of producing hemorrhagic purpura in white mice, rabbits, and guinea pigs.

2. The purpura-producing principle resists heating to 100°C. for 10 minutes; it resists oxidation; it is filter-passing; its activity is destroyed by digestion with trypsin; it is obtained from pneumococcus extracts by full saturation with $(NH_4)_2SO_4$, after the acetic acid-precipitable substances have been removed from the extracts.

3. The purpura-producing principle is common to all four types of pneumococcus and apparently bears no relation to virulence.

4. The purpura-producing principle is probably a degradation product of pneumococcus.

5. This principle is not associated with the hemotoxin of pneumococcus, since the hemolytic activity of an extract may be destroyed without effect on the ability to cause purpura.

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THE PRODUCTION OF PURPURA BY DERIVATIVES OF PNEUMOCOCCUS.

II. The Effect of Pneumococcus Extract on the Blood Platelets and Corpuscles.

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It has been shown (1) that pneumococcus extracts produce purpuric lesions in white mice, rabbits, and guinea pigs. A number of toxic substances (benzene, saponin, diphtheria toxin, etc.) also have the property of producing purpura in experimental animals. It appears from previous studies that a combination of factors is involved in the production of experimental purpura. One of them is the excessive diminution in the number of blood platelets, which may be due either to their actual destruction, or damage to their seat of origin; the other is injury to the capillary walls. It seemed of interest, therefore, to study the variations in number of the blood platelets in white mice which were injected with pneumococcus extract.

Method Employed in Counting Platelets.—It was not possible to count the platelets by the direct method on account of the small quantities of blood available in mice. The indirect method was therefore adopted, in spite of the errors inherent in it, and was found satisfactory in that it gave a conception of the approximate number of blood platelets present.

The mouse's tail was warmed over an electric bulb to insure a free flow of blood. The tail was then laid on a block of wood with the tip immersed in normal salt solution containing 2 per cent sodium citrate. A short piece of the tail was cut off with a razor blade, and as the blood began to flow, the tail was removed from the citrate solution, and touched to a clean glass slide. The drop was spread over the surface with the edge of another slide. Four slides were made for each count. Haste to prevent clumping of the platelets was not necessary because of the presence of the anticoagulant. More blood was taken for erythrocyte and leucocyte counts and the bleeding was stopped by searing. The slides for the platelet counts were stained with Wright's stain in the usual manner. After

the relative number of blood platelets and erythrocytes had been determined from the stained smears, and the actual number of erythrocytes had been found, the number of blood platelets was determined by proportion.

Care was exercised to prevent an unnecessary amount of bleeding. Control counts after similar repeated bleedings in normal mice showed no marked changes referable to the loss of blood.

EXPERIMENTAL.

The average number of erythrocytes in normal mice was found to be 8,000,000 and the platelets 1,800,000 per c.mm.

Effect on Platelets.—The mice were injected intraperitoneally with doses of 0.3 to 0.6 cc. of extracts obtained from Group IV, Type II, Type III, and the variant form of Type I pneumococcus. The method of preparation of these extracts has been described by Avery and Neill (12). All gave similar results. Counts were made at varying intervals, from half an hour to several days. Variation in the platelet count was observed in twenty mice.

A rapid fall in the number of platelets occurred soon after the injection of the extract. In one instance a third of the total number disappeared within 20 minutes. Usually the greatest diminution occurred after 24 hours. The lowest count obtained was about 8000 per c.mm. The number increased subsequently in the mice that survived the effects of the extract and reached normal in from 4 to 9 days. Thereafter there was an increase above normal lasting 2 to 12 days and by the 14th to the 20th day there was a return to normal which was maintained. In mice which failed to show purpura the platelets did not fall below 800,000 per c.mm. As a rule, purpura occurred only when their number was less than 500,000 per c.mm. Some specimen results are recorded in Tables I and II and illustrated by Graphs 1 and 2.

In general, the curves obtained by plotting the number of blood platelets in white mice, following the injection of pneumococcus extract, were similar to those obtained during the course of pneumonia, a number of other infectious diseases, and following the injection of certain toxic substances or antiplatelet serum in experimental animals. The initial reduction in the number of blood platelets was followed by a rise which exceeded the normal count, and later returned to normal.

In order to determine whether a cumulative effect on the platelets

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could be obtained, one mouse was given five daily injections of small doses of the extract (0.05 cc. daily). During the course of these injec-

TABLE I.

Changes in Platelets and Erythrocytes Following Injection of Pneumococcus Extract.

Mouse III injected intraperitoneally with 0.4 cc. of Type II extract.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.....	1,500,000	8,130,000	
20 min. after injection.....	1,000,000		
1 hr. " "	700,000		
2 hrs. " "	510,000		Slight purpura in right ear.
4 " " "	500,000	5,240,000	Purpura in feet and tail also.
24 " " "	160,000	4,300,000	Purpura in feet, tail, and ears.
52 " " "	140,000	4,300,000	" " "
96 " " "	240,000	2,400,000	Purpura disappearing.
108 " " "	810,000	2,240,000	No purpura observed.
192 " " "	2,000,000	2,900,000	
9 days " "	2,800,000	4,100,000	
12 " " "	2,100,000	4,700,000	
18 " " "	1,600,000	7,300,000	

TABLE II.

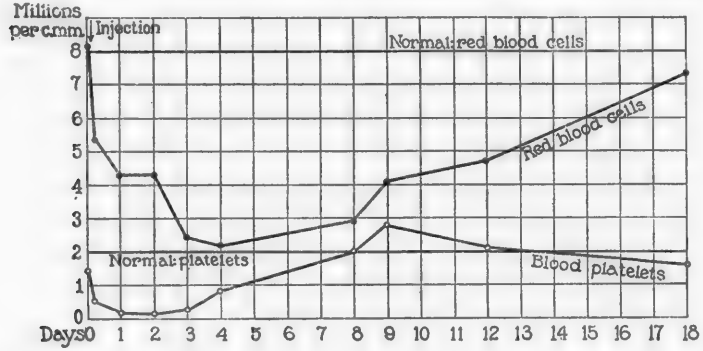
Changes in Platelets and Erythrocytes Following Injections of Pneumococcus Extract.

Mouse VI injected with 0.4 cc. Group IV extract.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.....	2,000,000	8,200,000	
90 min. after injection.....	1,400,000		
4 hrs. " "	640,000		Purpura in right ear.
24 " " "	74,000	5,800,000	Severe purpura in both ears, feet, and tail.
72 " " "	750,000	4,200,000	Purpura disappearing.
5 days " "	3,000,000	5,900,000	Slight trace of purpura left.
9 " " "	5,700,000	8,200,000	
14 " " "	3,700,000	6,500,000	
21 " " "	2,000,000	8,000,000	

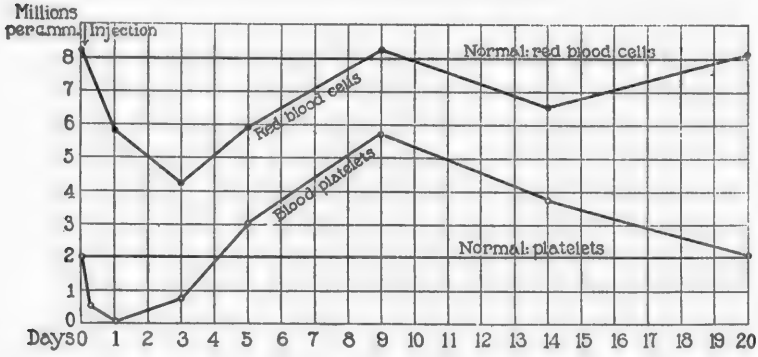
tions there was a gradual diminution in the number of blood platelets to 750,000 per c.mm., after which the number began to return toward the normal, when the experiment was discontinued.

Camera lucida studies did not reveal the variations in size of the platelets which have been observed during the course of pneumonia in man (2).


Millions per c.mm.
Injection
Normal: red blood cells
Red blood cells
Normal: platelets
Blood platelets
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

GRAPH 1. The effect of pneumococcus extract on the number of blood platelets and red cells. Graph illustrating Table I.

The mouse of 15 gm. was injected intraperitoneally with 0.4 cc. Type III extract.


Millions per c.mm.
Injection
Normal: red blood cells
Red blood cells
Blood platelets
Normal: platelets
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

GRAPH 2. The effect of pneumococcus extract on the number of blood platelets and red cells. Graph illustrating Table II.

The mouse of 15 gm. was injected intraperitoneally with 0.4 cc. Group IV extract.

Effect on Erythrocytes.—Injections of pneumococcus extract caused a marked reduction in the number of erythrocytes as well as in the number of platelets as shown in Tables I and II. The rate of dim-

inution was somewhat slower for erythrocytes, but the diminution continued even after the blood platelets had begun to increase in number. The reduction of erythrocytes was most marked in from 3 to 7 days, and there was a return to normal in 10 to 20 days. The diminution of erythrocytes usually amounted to 50 per cent or more. That this anemia was not entirely referable to hemorrhage into the tissues is concluded from the fact that anemia may follow injections of pneumococcus extract in the absence of a visible purpura. Conversely, severe purpura may develop without the occurrence of a severe anemia as will be shown further on.

An occasional mouse showed a marked lipemia following severe anemia. This has also been observed by Bloor (3) and Horiuchi (4), in experimental animals in which anemia had been induced artificially.

Effect on Leucocytes.—These cells did not show marked fluctuation. Frequently a slight leucocytosis was observed immediately after the injection of the extract, followed by a slight leucopenia.

Observations on the Lytic Action of Pneumococcus Extract.

The rapid diminution in the number of platelets and erythrocytes in the injected mice suggested that the destruction might be due to direct lysis of the cells. Accordingly the lytic effect of the extract was tested *in vitro*.

Human and rabbit platelets were obtained by rapidly running 30 cc. of blood into 3 cc. of saline solution containing 5 per cent sodium citrate. The mixture was centrifuged slowly for 5 minutes to sediment the red and white blood cells. The supernatant fluid was separated and centrifuged at high speed to obtain the blood platelets. The blood platelets and erythrocytes were washed separately twice, and each sediment suspended in saline. In performing the lytic tests, 0.5 cc. quantities of extract, suspensions of platelets, and erythrocytes were used.

It was found that the extract was lytic for platelets and erythrocytes in dilutions as high as 1:100 when incubated with them for 1 hour at 37°C. However, the lytic action was completely prevented when both platelets and erythrocytes were suspended in serum instead of salt solution. Blood platelets which had been suspended in serum and stronger concentrations of the extract appeared blurred when stained and examined under the microscope. No lytic action against leucocytes was demonstrated *in vitro*.

Similar observations were made when bile, saponin, or sodium oleate were employed as lytic agents. The bile we used was lytic for platelets in dilutions of 1:600, the saponin in dilution of 1:200,000, and the sodium oleate in dilution of 1:100,000. In the same or somewhat greater concentrations, however, none of these agents was lytic for platelets when suspended in serum.

Observations were made *in vitro* on the effect of heat on the thrombolytic activity of pneumococcus extracts. The extract was heated at 55°C. for 10 minutes; and titrations of it were made before and after heating. The results are shown in Table III.

TABLE III.

The Effect of Heated and Unheated Pneumococcus Extracts on Platelets and Erythrocytes in Vitro.

Dilution of extract.	Unheated extract.				Extract heated 10 min. at 55°C.			
	Platelets suspended in		Red blood cells in		Platelets suspended in		Red blood cells in	
	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.	Serum.	Salt solution.
1:1	−	+++	−	+++	−	−	−	−
1:10	−	++	−	++	−	−	−	−
1:50	−	+	−	+	−	−	−	−

+++ = complete lysis.
++ = incomplete lysis.
+ = slight lysis.
− = no lysis.

It will be seen that heat destroys the lytic activity *in vitro* of the extract for blood platelets and for red blood cells, as Cole (5) and Avery and Neill (6) have already shown. However, heat does not destroy the activity of the purpura-producing constituent. Heated extract when injected into white mice causes changes in number of blood platelets similar to those following the injection of unheated extract. But the number of red blood cells alters much less. Some figures are given in Table IV.

Adsorption Experiments.—Pneumococcus extract was brought in contact with blood platelets and erythrocytes to determine the possibility of a selective adsorption. The extract was mixed with one-third its volume of washed red blood cells which had been heated for

TABLE IV.

Changes in Platelets and Erythrocytes Following the Injection of Heated Pneumococcus Extract.

Mouse IV, injected with 0.4 cc. of extract which was heated for 20 minutes at 56°C.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.....	2,300,000	9,600,000	
1 day after injection.....	450,000	9,000,000	Marked purpura in both ears.
2 days " "	710,000	9,100,000	" " " " "
5 " " "	1,500,000	9,200,000	

TABLE V.

The Effect of Adsorption of Pneumococcus Extracts on Platelets and Erythrocytes in Vitro.

Extract adsorbed with	Action on red cells. Dilution of extract.			Action on platelets. Dilution of extract.	
	1:1	1:5	1:10	1:1	1:5
Red cells.....	−	−	−	−	−
Platelets.....	++	+	−	+	+
White cells.....	++	+	−	+	+
Kaolin.....	+++	++	++	+++	+
Unadsorbed.....	+++	++	++	+++	+

TABLE VI.

Changes in Platelets and Erythrocytes Following the Injection of Adsorbed Pneumococcus Extract.

Mouse X, injected intraperitoneally with 0.5 cc. of extract after adsorption with erythrocytes.

Time of examination.	No. of platelets.	No. of erythrocytes.	Remarks.
Before injection.....	2,000,000	8,400,000	
1 day after injection.....	170,000	8,800,000	Purpura after 18 hrs.
3 days " "	840,000	3,100,000	

10 minutes at 70°C. Specimens of the material were kept both in the ice chest and in the incubator for ½ hour. The extract treated in this manner was no longer lytic for either erythrocytes or platelets. Extracts similarly treated with blood platelets showed a lesser diminution in hemolytic and thrombolytic titer. (See Table V.)

The extracts submitted to treatment of the sort described were still capable of producing purpura. Moreover, extracts from which the hemolysin had been adsorbed with red cells still produced marked anemia when injected into mice. Table VI shows these findings.

Mixture of the extract with erythrocytes at ice box temperature resulted in a binding of the lysin without hemolysis. The red cells upon which was fixed the lysin, when washed thoroughly and suspended in cold saline, hemolyzed completely when warmed to room temperature.

DISCUSSION.

On the whole, the behavior of the blood elements after the injection of pneumococcus extract is similar to the effects observed after the injection of other toxins.

Bunting (7) found that when saponin is given intravenously to rabbits a destruction of platelets and erythrocytes occurs and there is a lesser injury to the leucocytes. Saponin also causes destruction of the cells in the bone marrow and in particular it causes an injury to the capillary walls resulting in extensive hemorrhages. Bunting found that the changes in the number of platelets follows a curve independent of those of the other blood elements.

Duke (8, 9) showed that diphtheria toxin and benzene in large doses are poisonous to the bone marrow and cause a fall in the number of platelets and red cells. He thinks that the platelets themselves are also affected by these agents and that this factor contributes to the diminution in numbers. He believes that the rapidity of the changes can be accounted for by assuming that the platelets normally are short lived bodies.

Our results seem to corroborate these views inasmuch as the evidence indicates that the diminution in the number of platelets and erythrocytes is brought about by some action other than direct lysis alone. The lytic action is prevented *in vitro* by heating the extract and the heated extract still causes purpura. Moreover, lysis by unheated extracts is inhibited by serum. Adsorption of pneumococcus extract with blood platelets or red cells renders the thrombolysin inactive *in vitro*; but this procedure does not prevent the extract from producing purpura and thrombopenia in white mice. The lesions were as extensive as those caused by unabsorbed extract. If the diminution in the number of platelets were due to lysis alone, the number would return to normal in a much shorter time than is the case. In instances

such as those described by Richardson (10) and Bunting in which bleeding was done to lower the number of blood platelets in rabbits, the number returned to normal in 2 or 3 days. Duke (8) has shown that when platelets are almost completely removed from the circulation in dogs by defibrinating blood and reinjecting it, the number returns to normal in 3 to 5 days. However, if toxic agents which poison the platelet-forming organs are injected, the rate of regeneration is markedly retarded.

The rapid diminution in number of platelets after the injection of pneumococcus extract seems to point to a direct action on the platelets in the circulation, while the slow rate or regeneration points to a toxic effect of the extract on the formation elements in the bone marrow. A few mice did not show purpura until 2 or 3 days after the injection. There may have been a delayed action on the circulating platelets; or, possibly a toxic action on the marrow which prevented the normal formation of platelets, until there were so few left that purpura resulted. Students of purpura know that injury to the capillary walls occurs. Intact vessels will probably not permit leakage, no matter how low the platelet count may be. Degkwitz (11) contends that the normal function of platelets is to plug up interstices between capillary endothelial cells. Should this be the case then primary injury to capillary walls need not occur.

The pathology of the purpuric lesions is being studied and search is under way for changes in the hemopoietic centers and capillary walls. Avery and Neill (unpublished work) have determined that the activity of pneumococcus extracts *in vitro* may be destroyed by heat and oxidation, but the hemolysin may still function as an antigen. Our data add evidence to the complicated nature of the hemolytic activity of pneumococcus extract.

SUMMARY.

A study has been made of the variation in number of the blood platelets, and the red and white blood cells of white mice injected with pneumococcus extract. The blood platelets were greatly diminished after the injection, the greatest decrease usually occurring after 24 hours. Purpuric lesions usually developed when the number of blood platelets became less than 500,000 per c.mm. Regeneration of

the platelets was accomplished by the 4th to the 9th day but there was an overregeneration and the return to normal did not take place until 2 weeks had elapsed.

The red cells were also greatly reduced in number, but the rate of their destruction and regeneration was somewhat slower than that of the platelets. The leucocytes were slightly if at all influenced by the pneumococcus extract.

Pneumococcus extracts were shown to be thrombolytic and hemolytic. Heat destroyed the activity of both the lysins *in vitro*. Heated extract produced purpura in mice but did not cause a severe anemia. Extracts adsorbed with either blood platelets or red blood cells showed a marked diminution in their thrombolytic and hemolytic activity *in vitro*. Such extracts, however, produced purpura as well as severe anemia and thrombopenia in mice.

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SEROLOGICAL RELATIONSHIPS OF TYPE-SPECIFIC AND DEGRADED PNEUMOCOCCI.

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In a previous paper (1) it was shown that a strain of Pneumococcus Type I loses its specificity and virulence when subjected to certain unfavorable cultural environments. Yoshioka (2) has brought about a similar process of degradation in strains of Pneumococcus Types II and III. From these studies it is apparent that at least two general and widely different forms exist among pneumococci of the fixed types (Types I, II, and III), one comprising the virulent, type-specific, so called S strains, and the other including the avirulent, non-specific, so called R strains.

Both Griffith (3) and Yoshioka found that antisera prepared by using R strains for immunization did not agglutinate the virulent type-specific organisms and did not protect against infection caused by them. On the other hand, R strains were agglutinated by R sera and also by type-specific antipneumococcus sera regardless of type.

Type-specific pneumococci are capsulated and capable of elaborating the type-specific soluble substances which have been identified by Avery and Heidelberger as the carbohydrate constituent of the pneumococcus cell (4). Pneumococci of the R forms are not capsulated and do not elaborate the soluble substances which endow the cell with type specificity. In sequence with the recent observations of Avery and his associates (5–7), on the antigenic properties of the cellular constituents of pneumococcus, it seemed of interest to study further the immunological relationships between the so called S and R forms of pneumococci.

Methods.

Preparation of Sera.—Type I, II, and III pneumococci were repeatedly grown in broth containing dilutions of immune serum of the respective type, until upon

plating the cultures only colonies of the rough, avirulent R form appeared. From ten to twenty transfers in 1 per cent immune sera were necessary. Antipneumococcus sera were then prepared with the three type-specific S strains and were called Type I, II, and III S sera, and also with the variant R strains of their respective types which were designated Type I, II, and III R sera.

For the preparation of immune sera, the bacteria from young, actively growing cultures were suspended in salt solution and immediately killed at 56°C. to minimize the amount of autolysis or cell solution. Autolysis is undesirable since Avery and Heidelberger (7) have found that dissolution of the bacterial bodies is accompanied by dissociation of the specific antigenic complex and consequent loss of power to provoke type-specific antibodies. Rabbits were immunized according to the method of Cole and Moore (8) by the intravenous injection of pneumococci of the three specific types and of the variant strains derived from them by methods already described.

Sera were absorbed by mixing 10 per cent dilutions of the homologous serum with definite volumes of pneumococcus bodies obtained by centrifuging heated broth cultures. For instance, 0.2 cc. of the bacterial sediment was emulsified in 2 cc. of the diluted serum. The mixture was incubated for 2 hours at 37°C. and placed in the ice box overnight. The bacteria were then removed from the serum by centrifuging. R sera usually required a second exposure with one-twentieth volume of pneumococci for complete absorption.

Autolysates of the pneumococci were obtained by suspending the bacterial sediment from 150 cc. of an 8 hour dextrose broth culture in 10 cc. of salt solution and allowing the cells to disintegrate at 37°C. for 24 to 48 hours or until autolysis was nearly complete. The centrifuged supernatant fluids or autolysates were faintly opalescent. Solutions of pneumococci in bile did not give clear-cut reactions and were not used.

The pneumococcus protein and the anti pneumococcus-protein serum were prepared according to the methods of Avery and Heidelberger (4) and Avery and Morgan (5).

Considerable difficulty was encountered owing to the rapid autolysis and spontaneous agglutination of the II R and III R strains when used for agglutination tests. This was overcome to a large extent by transferring the strains eight or ten times in plain broth and finally killing by heat. Suspensions of heat-killed pneumococci were used in all agglutination tests.

Experiments with Antipneumococcus Serum.—The antipneumococcus serum (S) prepared by immunization with intact cells of the type-specific, virulent form contains predominantly an antibody reactive only with the soluble specific substance of the homologous type. However, since cultures may contain both intact cells and a variable amount of degraded cells as well as products of cell dissolution (7), and since in the material used for immunization dissociation is

TABLE I.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum.

Agglutination.					Precipitation.		
Bacterial suspensions.	Antipneumococcus serum (S) (Type I).			Normal rabbit serum.	Bacterial solutions.	Antipneumococcus serum (S) (Type I). 1:10	Control normal rabbit serum. 1:10
	1:10	1:20	1:40	1:10			
Type I–S pneumococci.....	++++	+++	+	−	Type I specific substance 1:40,000.....	++++	−
" II–S "	−	−	−	−	Type I–R autolysate.....	++	−
" I–R "	++	+	+	−	" II–R "	++	−
" II–R "	+	+	+	−	" III–R "	++	−
" III–R "	++	+	+	−	Pneumococcus protein solution 1:300.....	++	−
					Control salt solution.....	−	---

++++ = firm disc.
+++ = disc easily broken up.
++ = coarse agglutination or heavy precipitate.
+ = fine agglutination or light precipitate.
− = no agglutination or precipitation.

bound to occur in spite of precautions to prevent it, some of the cell protein becomes free and acting independently of the antigenic complex of the intact cell gives rise to an antiprotein immune body which is species-specific and distinct from the type-specific antibody. The usual antipneumococcus sera apparently contain both forms of antibodies.

It has been found on previous occasions that all R strains as well as the protein of pneumococci react in immune S sera regardless of type derivation. On the other hand, type-specific pneumococci (S forms) agglutinate only in their corresponding type sera. Agglutination and precipitin reactions of Type I-S antipneumococcus serum with suspensions and autolysates of various pneumococci are shown in Table I.

In Table I evidence is presented that Type I antipneumococcus serum contains specific agglutinins for homologous S strains, and precipitins for the homologous specific substance. It is not reactive with pneumococci of heterologous specific types. However, in addition to the type-specific antibodies there are present agglutinins for cells of the R forms, regardless of type derivation, and precipitins which react with autolysates of R strains and with solutions of pneumococcus protein. It would seem, therefore, that antipneumococcus serum contains the type-specific antibody together with antibodies which are common to all R strains and pneumococcus protein.

If antipneumococcus serum of this nature is absorbed with organisms of a homologous S type, it is possible to remove the type-specific antibody and leave the protein antibody intact. Table II shows the reactions obtained with a serum so treated.

It is apparent that neither the homologous S strain nor soluble specific substance derived therefrom is precipitated by the absorbed serum. The agglutinins and precipitins common to the R strains, however, remain, although somewhat diminished in titre. It is assumed that the diminution in titre is due to the absorption of some of the non-specific antibodies by some degraded forms or products of autolyzed pneumococci which appeared in the S culture during the preparation of the suspension for absorbing.

Just as it is possible to absorb the type-specific antibody from antipneumococcus serum and leave the protein antibody, so it is

likewise possible to absorb the latter and leave the former antibody practically undiminished in titre. Table III shows the serological

TABLE II.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum Absorbed with Type I-S Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci.....	−	−	−	Type I specific substance 1:40,000.....	−
" I–R "	+	+	+	Type I–R autolysate.....	+
" II–R "	+	+	−	" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300.....	+
				Control salt solution.....	−

TABLE III.

Agglutination and Precipitin Reactions with Type I-S Antipneumococcus Serum Absorbed with Type I-R Pneumococcus.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci.....	++++	+++	+	Type I specific substance 1:40,000.....	++++
" I–R "	−	−	−	Type I–R autolysate.....	−
" II–R "	−	−	−	" II–R "	−
" III–R "	−	−	−	" III–R "	−
				Pneumococcus protein solution 1:300.....	−

behavior of a type-specific serum absorbed with an R strain of pneumococcus.

Absorption of Type I antipneumococcus serum with a strain of

the degraded R forms derived from the same type leaves the titre of the type-specific antibody unaffected. Both the agglutinins for the R forms of pneumococci and the precipitins for autolysates and pneumococcus protein are completely removed, leaving a serum reactive only with homologous Type I-S pneumococci and soluble specific substance derived from them.

The experiments cited above have been repeated and results similar to those for Type I serum have been obtained with antipneumococcus sera and the corresponding organisms of Types II and III.

Several attempts were made to produce a serum *in vivo* containing only the type-specific antibody. The usual procedure of immunization was employed with the exception that for each injection organisms were obtained directly from an infected mouse. The mouse was inoculated intraperitoneally with pneumococci and killed in 12 hours. The peritoneal cavity was washed with saline and the suspension immediately centrifuged, washed once, and heated at 56° for ½ hour. By this rapid procedure it was hoped that autolysis with its coincident dissociation of the carbohydrate and protein constituents of the bacterial antigen would be eliminated. However, after immunization of six rabbits by this method it was found that each serum contained varying traces of the non-specific protein antibody, although but slight in amount when compared with antipneumococcus sera prepared in the usual way.

Experiments with Anti-R Pneumococcus Serum.—Since no type-specific substances are elaborated by the R strains it seemed likely that sera prepared against these strains would not contain any of the specific antibodies, but only those reacting in common with all R strains. Agglutination and precipitin reactions against specific and degraded strains of pneumococci by an anti-R serum are shown in Table IV.

It is evident from Table IV that the anti-R serum does not contain any type-specific agglutinins for the S strain from which the R form was derived. Neither does it contain precipitins for the homologous soluble specific substance. Agglutinins are present, however, for both homologous and heterologous R strains. There are also precipitins for pneumococcus autolysates of all types, both S and R. The autolysates of the S strains obviously contain the free specific element

and the free protein, but the latter alone is reactive in R serum. By virtue of its common nature, the protein of each autolysate is therefore

TABLE IV.

Agglutination and Precipitin Reactions with Type I-R Serum.

Agglutination.				Precipitation.	
Bacterial suspensions.	Serum dilutions.			Bacterial solutions.	Serum dilution. 1:10
	1:10	1:20	1:40		
Type I–S pneumococci.....	−	−	−	Type I specific substance 1:40,000.....	−
" I–R "	++	+	+	Type I–S autolysate.....	+
" II–R "	++	+	+	" II–S "	+
" III–R "	++	+	+	" III–S "	+
				" I–R "	+
				" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300.....	+

TABLE V.

Agglutinanon and Precipitin Reactions with Type I-R Serum Absorbed with Type I-R Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilutions. 1:10
	1:10	1:20	1:40		
Type I–R pneumococci.....	−	−	−	Type I–S autolysate.....	−
" II–R "	−	−	−	" II–S "	−
" III–R "	−	−	−	" III–S "	−
				" I–R "	−
				" II–R "	−
				" III–R "	−
				Pneumococcus protein.....	−

reactive. Similarly, pneumococcus protein, regardless of the form from which it is isolated, is precipitated by the R serum.

It is possible to absorb the antibodies from an R serum with an R

strain. This procedure removes the agglutinins for the R strains and the precipitins for the pneumococcus protein, leaving the serum inactive. The agglutination and precipitin reactions of a Type I-R serum absorbed with a Type I-R strain are given in Table V. The absorbed serum being inactive does not agglutinate suspensions of the R organisms or precipitate pneumococcus protein either in its purified isolated form or as it occurs in cell autolysates.

Protocols similar to Table IV were obtained by using II-R and III-R sera. Reactions similar to those shown in Table V were obtained

TABLE VI.

Agglutination and Precipitin Reactions with Anti Pneumococcus-Protein Serum.

Agglutination.			Precipitation.	
Bacterial suspensions.	Serum dilutions. Anti pneumococcus-protein serum.		Bacterial solutions.	Serum dilution. 1:10
	1:10	1:20	1:40	
Type I–S pneumococci.....	−	−	−	Type I specific substance 1:40,000.....	−
" I–R "	++	+	+	Type I–S autolysate.....	+
" II–R "	+	+	+	" II–S "	+
" III–R "	+	+	+	" III–S "	+
				" I–R "	+
				" II–R "	+
				" III–R "	+
				Pneumococcus protein solution 1:300.....	+

with a III-R serum absorbed with a II-R strain, and with a I-R serum absorbed with a III-R strain of pneumococci.

Experiments with Anti Pneumococcus-Protein Serum.—Because of the consistent similarity in precipitin reactions of the pneumococcus autolysates, and of the prepared pneumococcus protein in the foregoing experiments, it seemed of interest to observe the serologic behavior of a serum prepared against this protein. The agglutination and precipitin reactions of an anti pneumococcus-protein serum with S and R strains are shown in Table VI.

It is significant that the reactions in Table VI resemble those shown in Table IV. Apparently sera prepared with R strains and with pneumococcus protein have similar serologic properties. The antiprotein element in the serum obviously being common to the protein of all types of pneumococci, both S and R, causes a precipitate in all of the autolysates and agglutinates suspensions of all R strains. The intact S organisms are not affected.

The similarity between the antiprotein serum and the anti-R serum is further demonstrated by the absorption of the agglutinins in the antiprotein serum with R strains. The results are tabulated

TABLE VII.

Agglutination and Precipitin Reactions with Anti Pneumococcus-Protein Serum Absorbed with Type I-R Pneumococci.

Agglutination.				Precipitation.	
Bacterial suspensions.	Absorbed serum dilutions.			Bacterial solutions.	Absorbed serum dilution. 1:10
	1:10	1:20	1:40		
Type I–R pneumococci.....	—	—	—	Type I–S autolysate.....	—
" II–R "	—	—	—	" II–S "	—
" III–R "	—	—	—	" III–S "	—
				" I–R "	—
				" II–R "	—
				" III–R "	—
				Pneumococcus protein solution.....	—

in Table VII. The resemblance of Table VII to Table V is self-evident. The agglutinins for the R strains and the precipitins for the cell autolysates and pneumococcus protein have been removed, leaving an inactive serum.

DISCUSSION.

Avery and Neill (6) have shown that solutions of pneumococci have antigenic properties strikingly different from those of the intact cells themselves. When cell solutions are used for immunization, antibodies arise which react with the common protein alone. On the

other hand, when the uninjured bacterial cell is used for immunization, antibodies arise which react with the type-specific carbohydrate substance. By virtue of the common properties of the protein constituent of pneumococci, sera containing the antibody to this protein react with the protein from all types of pneumococci. Antiprotein sera obtained by immunization with dissolved pneumococci are similar in their serologic reactions to sera produced by the injection of the isolated protein.

The experiments recorded in the present paper show that sera prepared by immunization with pneumococci of degraded types (R strains) resemble in their reactions sera prepared by immunization with the cell protein alone. It would seem, therefore, that R pneumococci stimulate the formation of antibodies by virtue of the protein constituent of the cell body. Previous mention has been made of the absence of capsule formation and of the associated specific substance in the R strains. Consequently the R strains, devoid of the type-specific antigen, cannot stimulate the formation of antibodies reactive with the type-specific substances and therefore have only the antigenic properties of the isolated protein. Thus, on account of the common antigenic properties of the protein of all pneumococci it is not surprising that R sera cross-agglutinate with R strains regardless of type derivation, and precipitate the free protein in autolysates of S strains.

The results on the whole substantiate the deductions of Avery and Heidelberger (7) regarding the relationship of the carbohydrate and protein constituents of the pneumococcus cell.

Assuming that the loss in type specificity of an S strain after degradation to the R form is due to a loss in the capacity to elaborate the type-specific carbohydrate substance associated with the capsule, and that the R form is merely an S cell minus this factor, it is possible to interpret the immune mechanism involved according to the views of these authors. Applying these conclusions to the results of their study it is only necessary to substitute in their scheme (7, Fig. 1) the symbol for protein (P) by R representing an R cell. The type-specific cell would then be an S-R combination and the R cell, R alone.

The confusing cross-reactions occasionally encountered in typing

pneumococci with the usual diagnostic sera, and the cross-reactions occasionally noted in S cultures containing unusual numbers of degraded pneumococci, are thus in part explained.

CONCLUSIONS.

1. Immune sera prepared with the degraded or variant forms of pneumococci (R strains) are similar in their reactions to sera prepared with the protein or cell solutions of pneumococci. They contain antibodies reactive with the protein of all types of pneumococci, but no antibodies reactive with the type-specific substances.

2. Pneumococci of the variant or R form, regardless of type derivation, are serologically identical and have the antigenic characteristics of pneumococcus protein. They evoke the species-specific and not the type-specific antibodies.

3. Antipneumococcus sera obtained by immunization with S strains may contain species-specific antibodies in addition to those which are type-specific. Each kind of antibody can be removed separately from these sera by selective absorption with the R or S strains of pneumococci.

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THYMECTOMY IN THE RABBIT.

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PLATE 1.

(Received for publication, October 14, 1925.)

Despite extensive investigations, the effect of surgical removal of the thymus in animals is still somewhat uncertain. This uncertainty is due, in part, to the difficulty that has been experienced in distinguishing between effects of thymoprivia and effects that might be attributable to other causes, especially complications arising during or subsequent to operation, postoperative care of animals, and the use of methods that give varying degrees of success with respect to operative injury and complete removal of the gland.

In the course of studies that were being carried out in these laboratories, it became desirable to compare the behavior of thymectomized rabbits with that of normal rabbits and rabbits from which other glands had been partially or completely removed. This necessitated an investigation of the operation of thymectomy itself and the immediate or early results of removal of the thymus. It was necessary, first, to devise an operation that could be depended upon for complete removal of the thymus with a minimum loss of animals, and, second, to determine whether the thymus could be removed from rabbits of certain ages without producing any serious impairment of health, within specified periods of time, which would interfere with their use in other experiments. A preliminary report on one phase of this work has been published (1). The object of this paper is to report the early or immediate results of thymectomy, obtained by a method that was developed for use in connection with the experiments referred to above.

Methods.

As the success of any experimental study of thymoprivia hinges upon the operation, it will be described in detail.

Reviews of the various methods which have been recommended for removal of the thymus in animals may be found in the papers by Asher (2), Matti (3), and Park and McClure (4). The two principal types of operation are represented by the closed method of Friedleben (5), which was introduced in 1858, and the open or transthoracic operation suggested by Basch (6–9) in 1902. In the method of Friedleben, an incision is made at the base of the neck, exposing the upper poles of the thymus; these are seized with blunt forceps and the gland withdrawn from the chest without attempt at hemostasis. At best, complete ablation is not certain by this approach and mortality is high.

In the transthoracic operation of Basch, the mediastinum is exposed anteriorly by splitting the sternum and the gland is removed by dissection. Unfortunately, Basch left no detailed description of his technique. The method has been modified by MacLennan (10) for use in rabbits, by Ranzi and Tandler (11), who introduced the Sauerbruch chamber, and by Klose and Vogt (12–15) and Park and McClure (4) in using positive pressure anesthesia.

Method Used.—The method of operation employed by us is essentially that of Basch, adapted to the rabbit. All operations were done under ether anesthesia; at first, we used positive pressure apparatus for the prevention of pulmonary collapse but found that this measure might be omitted.

Regional Anatomy.—The thymus of the rabbit is a bilobed organ, flat and roughly triangular in shape. It lies almost entirely within the chest and occupies the anterior mediastinum, the lobes uniting along the midline. The base of the gland usually lies at the level of the fourth rib; the apex reaches through the upper thoracic strait and in the form of two separate strands follows the carotid arteries upwards a variable distance into the neck; the lateral margins of the lobes lie in contact with the pleuræ. Ventrally the thymus is confronted by the chest wall, from which it is separated only by an aponeurosis containing muscle fibres, the substernal extension of the pretracheal muscle layer. Behind the thymus at its lower third is situated the base of the heart covered by the pericardium, at its middle third the aortic arch, and behind its apex is located the great carotid trunk which bifurcates at this point to form the two common carotid arteries. The blood supply of each lobe is received at the lateral margin at the level of the first rib, the artery branching usually from the corresponding internal mammary, and the vein contributing to the subclavian vein (see Fig. 2). Occasionally the gland receives a branch underneath from the carotid artery. The intrathoracic portion of the thymus is encased in loose, cellular connective tissue, which can be made to separate easily, but in the neck the gland processes are held firmly attached to the adjacent vessels. Important nerve structures, as for instance the vagus and phrenic, lie well behind and lateral to the thymus and are not encountered in the operative field. There is one other structure to be noted. This is a vein of good size that runs transversely in a position just above the upper end of the sternum and superficial to the pretracheal muscle layer and connects the jugular veins.

Operation.—On the day of operation, the usual feeding should be omitted. The

fur over the neck and chest is clipped and the skin is shaved from the larynx to the ensiform, dried, and treated with alcohol.

Ether is given by the ordinary open cone method. The animal is draped with two towels wrung out of 1:10,000 bichloride solution, the margins meeting in the midline over the field of operation and widely overlapping elsewhere. The operator should work from the left side.

The incision is made in the midline from the level of the fifth rib to the thyroid cartilage, and the skin is excluded from the operative field by clamping its edges to the margins of the draping towels. The incision is then continued into the left pectoral muscle, following the margin of the sternum from the fifth rib upwards to the base of the neck, where the attachment of the left sternomastoid muscle to the sternum is also divided. This exposes the costal cartilages at their sternal extremities together with the adjacent intercostal muscles. The soft tissues of the neck are entered along the midline by incising between thumb forceps, exposing the trachea from the sternum to the larynx. During this procedure the transverse vein at the base of the neck is sought for, double clamped, and divided. The thorax is then opened as follows: The upper end of the sternum is elevated with thumb forceps and the tissues are separated from its under surface by blunt dissection for a distance of about 1 cm. This permits the blade of a pair of scissors to be inserted beneath the first left costal cartilage close to the sternum and this structure divided without injury to the underlying parts. Elevation of the sternum is maintained while the second, third, and fourth ribs and adjacent intercostal muscles are cut at the left sternal margin. Bleeding from the intercostal vessels is controlled with clamps. The extensive opening into the chest thus obtained is prevented from diastasis by the presence of the substernal aponeurosis, and this must be divided with scissors. The incision may then be widely opened and the anterior mediastinum brought to view (see Fig. 1).

Before proceeding further, preparations are made for the closure of the chest. For this purpose a heavy silk suture is passed about the tip of each cut rib and tied as illustrated in Fig. 1. Care should be taken to avoid piercing the internal mammary vessels. Sutures are also passed and tied around the sternum just above each rib stump. One end of each suture is left long after tying, to form a leash of four threads attached to the bony framework on each side of the thoracic incision. These serve temporarily as retractors.

Removal of the thymus is now begun, commencing at the apex. One of the tongue-like processes of the gland in the neck is grasped with thumb forceps and separated by blunt dissection from the corresponding carotid artery as far downwards as the aortic arch. The other apical tag is freed in the same way (see Fig. 2). This dissection is somewhat difficult because of firm attachments and must be done with care, and one should watch for the glandular vessels that occasionally spring from the carotids. Then the base of the thymus is located in the pericardial fat; its edge is picked up in the midline, and by drawing upwards upon it the gland is peeled away readily, and without dissection, from the pericardium. A tunnel is now burrowed beneath the thymus from top to bottom, and through this is

passed a thread to act as a retractor. Separation of each lobe margin from its pleural attachment is now undertaken. The lower edge of a lobe is grasped near to the pleura with fine mouse tooth thumb forceps and separated by gentle traction (see Fig. 2). The pleural leaf in the rabbit is exceedingly delicate and will rupture at the touch of an instrument. All dissection must be avoided and separation accomplished by traction alone, although it is of advantage at times to wipe the pleura gently away from the gland with a small wet gauze sponge. A steady pull upon the lobe margin serves to anchor the gland, and the respiratory undulations and cardiac pulsations furnish sufficient pull to disengage the gland from the pleura. 3 or 4 minutes time may be required for each side. Should the pleura be injured, the rent must be stopped immediately by a wet sponge to prevent complete collapse of the lung. Once both lateral margins are free from the pleuræ, there remains only the hilus of each lobe to be dealt with. By blunt dissection, the fatty tissue in the region of the first rib on either side is cleared away and the glandular vessels brought to view (see Fig. 2). With these secured the gland may be removed.

Closure of the incision is done in three tiers. The chest wall is brought together by tying corresponding ligature ends; the divided muscles are united by a running, heavy silk suture; and the skin margins are approximated with a fine silk suture. No dressing need be applied.

Material.

The operation described above was performed on 50 male rabbits; of these 36 were between 1 and 2 years of age, 8 were 3 years old or more, and 6 were between the ages of 4 and 6 months. 36 of the rabbits that survived operation were subsequently inoculated with tumor or *Treponema pallidum;* the others were held for control observations. The period of observation prior to inoculation was from 10 to 22 days, in most instances 2 to 3 weeks; 29 of the 36 rabbits survived the inoculation and permitted observation over a period of from 2 to 7 months. The majority of the uninoculated rabbits were observed for approximately 3 months.

RESULTS.

Operative Results.— Among the 50 rabbits on which thymectomy was performed there were 5 deaths related immediately or remotely to the operative procedure. 1 of these was due to air embolism, and the others to shock. In our last series of 29 operations there was only 1 death; this occurred in an old animal. Pneumothorax occurred

on the right side in 14 cases, on the left in 4 cases, and bilaterally not at all. There were no ill effects from the pneumothorax.

The chest wall incision united firmly in all of the animals that survived the operation with very little deformity of the thoracic framework and no interference with the function of the fore legs.

Postmortem examination showed that removal of the thymus was complete in all but one animal so far as could be determined, without resorting to microscopic examination. In one rabbit, a small nodule of thymic tissue was found high in the neck; this may have been overlooked at the time of operation. If any fragments were left, they showed no appreciable tendency to hyperplasia. The thymic bed was obliterated by firm fibrous adhesions between the pericardium and the chest wall and the lungs were expanded to their normal proportions.

Postoperative Complications.—Wound infection occurred in 8 animals. Usually an abscess developed within the mediastinum. These infections became encapsulated and pursued a chronic, indolent course which in most cases did not appear to affect the general health of the animals. There were no pulmonary complications, except in the preliminary operations in which intubation of the trachea was done for positive pressure anesthesia. Several of these animals developed pneumonia, atelectasis, or empyema.

Effects of Thymoprivia.—Our study of the effects of thymoprivia was limited to observations on the general physical condition and the reaction of operated rabbits to disease. The results of the latter investigation will be reported elsewhere.

Removal of the thymus produced no appreciable effect on the general health and state of nutrition of the animals. Recovery from the operation was prompt and the operated rabbits showed a gain in weight or a maintenance of weight comparable in all respects to intact normal rabbits. A number of these animals were observed over a period of from 2 to 7 months but during this time no difference in the appearance and physical condition of thymectomized and of normal rabbits was detected. The behavior of young, adult, and old animals which had been operated upon was essentially the same.

DISCUSSION AND CONCLUSIONS.

Thymectomy in the rabbit is a comparatively simple procedure. The open method described above offers many advantages over any of the closed methods and success depends largely upon the observance of a few precautions; chief among these is the avoidance of haste and the observance of aseptic technique.

The operative mortality in our series of rabbits was 10 per cent and there was an additional loss of useful animals, due to secondary complications, amounting to 16 per cent. Among the last 29 rabbits, however, there was only 1 operative death, in an old animal, and 2 cases of slight infection. It appears, therefore, that with some experience the operation can be carried out successfully in nearly all animals, especially so if comparatively young rabbits are used. Old rabbits present greater difficulties on account of the presence of dense fibrous tissue within the field of operation.

While the animals of this series showed no immediate symptoms or alteration in physical condition that could be referred to thymoprivia, it is not to be assumed that removal of the thymus is entirely without effect even on rabbits of mature age. Our observations on the reaction of thymoprivic rabbits to disease show that this is not the case and that in all probability decided effects can be demonstrated if the problem is approached on another basis. The object of the present investigation was, however, to determine whether the thymus could be completely removed from rabbits of certain ages without producing any serious disturbance of health which would interfere with their use in other experiments or complicate the interpretation of results. It was found that this could be done.

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EXPLANATION OF PLATE 1.

FIG. 1. Thymus exposed preparatory to removal.
FIG. 2. Technique employed in removal of the thymus.

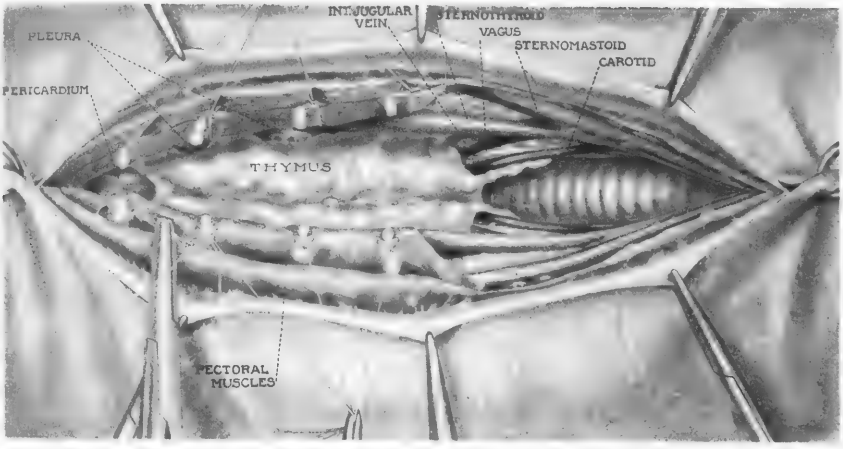

FIG. 1.

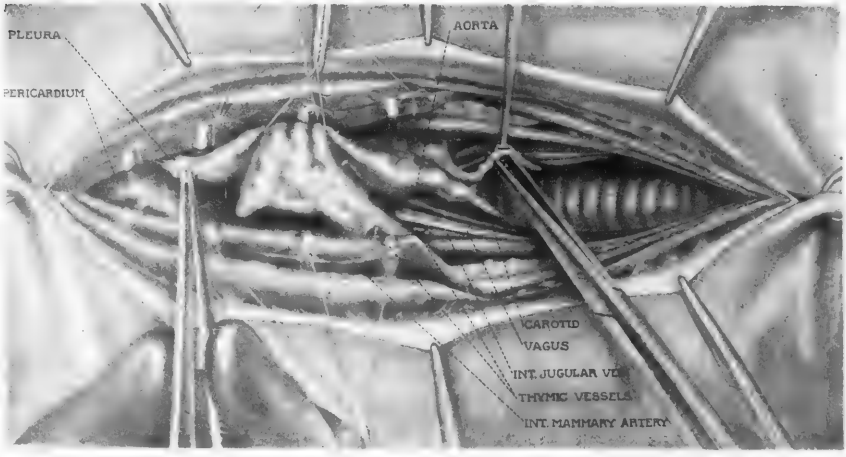

FIG. 2.

(Van Allen: Thymectomy in rabbit.)

UNIV. OF MICH.

ON THE SURFACE COMPOSITION OF NORMAL AND SENSITIZED MAMMALIAN BLOOD CELLS.

By STUART MUDD, M.D., and EMILY B. H. MUDD.

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Plates 2 and 3.

(Received for publication, October 2, 1925.)

A simple method has been described for observing the behavior of bacterial or other cells in the boundary surface between two immiscible liquid phases.[1] A theoretical discussion of this behavior has been given, based upon the equilibrium of interfacial tensions involved, and experimental observations concordant with the theory have been adduced.[2] The present communication describes the application of this method to normal and sensitized mammalian blood cells. Striking differences are found in the behavior of erythrocytes after sensitization and of leucocytes after exposure to heat. The results obtained permit certain inferences as to the composition of the cell surfaces and the mechanism of the reactions involved.

Method.

A drop of oil is drawn across a carefully cleaned slide. A small drop of dilute blood cell suspension is drawn along the slide a short distance from and at right angles to the streak of oil. One end of a clean oblong cover-slip is touched to the slide and to the oil so that the oil wets the under surface of the cover-slip along one end (Text-fig. 1). The other end of the cover-slip is now lowered onto the slide, thus spreading the oil into a film under one side of the slip and the blood into a film adjoining it. In the best preparations the blood film does not cover quite all of the area under its end of the slip.

When viewed with the dark-field microscope the interface between the films appears as a bright line with interference fringes, the red cells have their familiar

[1] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633.

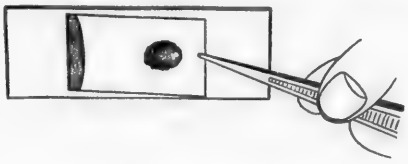
[2] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 647.

dark-ground appearance as shining discs or spheres, and the leucocytes appear as round or irregular cells packed with shimmering granules. To examine them both the Bausch and Lomb dark-ground condenser with substage 12 candle power bulb and the Zeiss improved paraboloid light- and dark-field condenser with carbon arc have been used satisfactorily. The Zeiss special apochromatic objective X is satisfactory.

Behavior of Red Cells.

The oil phase ordinarily advances slowly, pushing the aqueous phase ahead of it. As the interface thus overtakes the suspended or sedimented elements, a characteristic and distinctive behavior of the red and white cells is to be noted.

The erythrocytes are quickly taken into the interface and there, if freely suspended, they are pulled by the liquid-liquid tension into a characteristic lens shape (Fig. 1, *a*). Unless the interface is advancing



TEXT-FIG. 1.

too rapidly the freely suspended red cells often slide along the interface for a short distance before entering the oil. Stability of normal erythrocytes in the interface is, however, slight; with the aid of such little mechanical force as is afforded by the slow advance of the interface, they pass readily into the oil phase where they are commonly invisible unless adherent to the glass (Fig. 1, *b*). Escape from the interface is almost invariably into the oil, rarely into the water. The surface composition of the unsensitized erythrocytes is evidently such that they are more readily miscible with oil than with the aqueous phase but considerably less oil-miscible than are the acid-fast bacteria.[2]

If the liquid-liquid interfacial tension be designated T_{ow}, the cell-oil (solid-organic phase) tension T_{so}, and the tension in the cell-aqueous phase interface, T_{sw}, following the terminology of an earlier paper,[2] it is clear that for unsensitized erythrocytes T_{sw} is greater than T_{so}. It appears furthermore that neither solid-

liquid tension is greater than the sum of the other solid-liquid plus the liquid-liquid tension, but that the inequality $T_{sw} < T_{so} + T_{ow}$ is small, in some cases almost zero.

These relations explain the observation of Dautwitz and Landsteiner[3] who shook blood cell suspensions with toluene and, on separation of the phases, found the stromata in the toluene phase. The procedure of Dautwitz and Landsteiner might well form the basis of methods of separating a variety of particles with lipoid surfaces from aqueous suspending media.

The erythrocytes studied have been those of the four human groups, of the horse, donkey, sheep, dog, guinea pig, and rat. The test oils used have been Kahlbaum's triolein, tributyrin, and tricaprylin, olive, sperm, cod liver (Norwegian), mineral (Squibbs' Heavy Californian), and cedar oils, and melted vaseline (Chesebrough Manufacturing Co.). Dilute plasma and dilute serum, with and without free hemoglobin, 0.9 per cent NaCl, and Ringer-Locke solution have been used as the aqueous phase. Erythrocytes, freshly drawn or preserved several days, in the form of discs and spheres—more commonly the latter[4]—crenated forms, and stromata have been observed. The relationships described above have appeared regularly under these various circumstances throughout several hundreds of experiments.

Minor variations of course occur and would no doubt be much more in evidence were the method capable of finer quantitative discrimination and were a greater variety of substances used as organic phase. With the present technique, however, variations from the usual behavior appear to be small and for the most part difficult of formulation. The following may be noted.

Among the oils sperm and cod liver oil from another source wet the red cells with perceptibly less ease than the others. A phenomenon we may for brevity call "tails" is particularly likely to occur when the cells are suspended in plasma or when mineral oil is the organic phase. As the red cells in a slowly advancing interface in a thin film rub against the glass the region of contact tends to be drawn out into a process like the neck and stem of a pear provided the cells are sufficiently resistant to wetting by the oil; otherwise they snap over into the oil with very little deformation. These processes, or tails, have been most noticeable with normal cells in films made from plasma of sufficient

[3] Dautwitz, F., and Landsteiner, K., *Beitr. chem. Physiol. u. Path.*, 1907, ix, 448.

[4] *Cf.* Gough, A., *Biochem. J.*, 1924, xviii, 202; Ponder, E., *Quart. J. Exp. Physiol.*, 1924, xiv, 333.

concentration to clot on standing, but may be seen in less concentrated plasma and serum. The phenomenon is greatly accentuated with sensitized cells, as will appear later.

Red cells in dilute (0.5 to 5.0 per cent) plasma or serum may be pushed for some little distance before a very slowly advancing interface before entering the boundary surface at all. This phenomenon is probably due in part to the interface being occupied by adsorbed protein; it is accentuated in more concentrated sera (5 to 10 per cent). The increased difficulty of passage into and through the interface in concentrated serum and plasma is probably due also in part to condensation of serum proteins upon the red cell surface as shown by Coulter.[5]

Behavior of Sensitized Red Cells.

Red blood cells well sensitized with specific agglutinative and lytic anti-erythrocyte serum differ strikingly from the unsensitized cells in their interfacial reactions. Three phenomena either appear *de novo* or become accentuated with sensitization.

1. The sensitized red cells no longer pass readily from the interface into the oil phase. An interface may advance rapidly and few or no red cells cross from the boundary surface into the oil. Sensitized red cells often slide for long distances along the interface. If such cells do cross into the oil it is with obvious mechanical aid and ordinarily there occurs a perceptible deformation of the interface or of the erythrocyte surface or both.

2. The manifestation already referred to as tails. The sensitized cells agglutinate of course and tend to stick to the glass. As the interface moves by such cells or cell clumps, it tends to carry the cells with it since they resist wetting by the oil phase. The cells are often anchored by their points of contact with the glass. The result is that pear-shaped (Fig. 2) or filamentous (Figs. 5 and 6) prolongations of the red cell surfaces are produced. If the red cell is anchored by a larger area a dumb-bell shape may result; such a cell has even been observed to pinch in two, each half cell then rounding up.

This tail phenomenon is beautifully, though faintly, shown in the photographs of Fig. 5, but has been almost lost in reproduction. Comet-like streamers are just discernible stretching from the cell clump in the center of the field toward the lower right hand corner. From the single cell between the retreating interface (upper left hand corner)

[5] Coulter, C. B., *J. Gen. Physiol.*, 1921–22, iv, 403.

and the cell clump in the center, two tails forming an inverted U may just be seen pointed downward and slightly to the right (Fig. 5, *c*).

3. The interface is often bent backward in passing over very small cell clumps or even single sensitized cells.

Figs. 3, 5, and 6 are enlargements from moving picture films of such phenomena. The direction of advance of the interface is indicated in each case by the arrow. In Fig. 3 the advancing interface has been blocked locally by a clump of sensitized red cells adherent to the glass and has been bent backward into a right angle. In Fig. 5 a similar occurrence is carried a step further. The oil phase advancing on the aqueous phase (upper left hand corner) with its suspended blood cells meets groups of sensitized red cells it cannot easily wet. The interface, stopped locally, advances elsewhere and thus leaves a clump of agglutinated cells in aqueous salt solution isolated within the oil (Fig. 5, *a* and *b*); streamers behind the clumped cells indicate how far they have been dragged by the advancing interface from their original site of attachment to the glass. Still retarded by a small group of cells the interface is dragged over one or more of them (Fig. 5, *b* and *c*) and then retracts according to the law of minimal areas.[6] One erythrocyte with tails remains stuck to the glass and visible, the others are either suspended in the oil and hence invisible or have been carried out of the field on the aqueous side of the retracting interface. Fig. 6 shows a clump of agglutinated red cells as it is left behind by the retreating interface; tails are here more plainly shown.

Specific sensitization of the erythrocytes, then, stabilizes them in the interface between organic and aqueous phases; this effect is the result of an alteration of the red cell surfaces by the sensitizing agent such as to make them more difficultly miscible with oil. The predominantly non-polar[7] surface of the unsensitized erythrocyte thus becomes with sensitization more polar. In terms of interfacial tension, specific sensitization increases the inequality $T_{sw} < T_{so} + T_{ow}$ and decreases the inequality $T_{so} < T_{sw} + T_{ow}$. This observation is in harmony with that of Meyer[8] who found hemolytic amboceptors to be insoluble in lipoid solvents. The further significance of the alteration of the erythrocyte surface with sensitization will be discussed below.

This altered interfacial behavior, or "stabilization reaction" as

[6] Plateau, J., Statique des liquides soumis aux seules forces moléculaires, Paris, 1873, 9.

[7] Lewis, G. N., *J. Am. Chem. Soc.*, 1913, xxxv, 1448; 1916, xxxviii, 762. Harkins, W. D., in Holmes, H. N., Colloid symposium monograph, New York, 1925, ii, 163.

[8] Meyer, K., *Arch. Hyg.*, 1908, lxvii, 114.

it may be called, is less sensitive than lysis or agglutination. The red cells to be tested are washed several times with Ringer-Locke or physiological NaCl solution, mixed with the specific anti-erythrocyte serum in proper dilution, and allowed to stand in the ice box usually for an hour or more. Tested then the altered interfacial behavior has been found with the following concentrations of immune serum, the homologous red cells being each time in 1 per cent suspension: anti human-erythrocyte rabbit serum (obtained through the kindness of Dr. Hideyo Noguchi) 0.3 per cent and higher; anti horse-erythrocyte rabbit sera,[9] Nos. 32 and 40, 2.0 per cent and higher, Nos. 15 and 30, a few tenths per cent and higher.[10] Unwashed horse cells in anti-horse sera 16 and 31 gave some positive and some negative stabilization reactions in concentrations of 2 to 6 per cent. Titers with unwashed cells were not constant, however.

Cross-tests with these same immune sera do not yield the stabilization reaction. Thus the anti-human serum in 5 per cent concentration gave negative tests with 1 per cent horse cells. 1 per cent suspensions of human cells tested with 20 per cent anti-horse serum 40 and with 4.8 per cent anti-horse serum 15 were not stabilized. At approximately the same concentrations and at lower ones these sera gave strong positive reactions with homologous cells. Rat cells, 1 per cent, in 3 to 7 per cent anti pony-erythrocyte rabbit serum were not stabilized in the majority of experiments; but in three tests the results were weakly positive.

Through the kindness of Dr. K. Landsteiner and Mr. J. van der Scheer, it has been possible to carry a step further the analysis of the action of immune serum upon the erythrocyte surface. These investigators, using alcohol extracts of red cells as antigens, have obtained

[9] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1925, xli, 427. The numbers we have given the anti horse-erythrocyte sera are taken from this paper.

[10] The high titers found for Sera 15 and 30 as compared with those previously found with Nos. 32 and 40 were unexpected. The explanation may well have been in the difference in the solutions in which the test red cell suspensions were made; *i.e.*, physiological NaCl in the later and Ringer-Locke solution in the earlier experiments. The reaction of the Locke solution as we made it up was about pH = 8.7. Coulter (Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 513) has shown that considerably less sensitizer is combined with the red cells at alkaline reactions than at the optimal weakly acid reaction.

anti-erythrocyte sera with a ratio of the lysin to agglutinin titers of about 1:1–1.5 instead of 1:4–8 as with sera obtained after injection of whole erythrocytes.[9] Two pair of sera, with one extract immune serum and one whole erythrocyte immune serum in each pair, have

TABLE I.

Organic phase.	Cell concentration.	Serum concentration.	Cells stabilized by serum.		Remarks.
			No. 40. Extract immune serum.	No. 32. Whole red blood corpuscle immune serum.	
	per cent	*per cent*			
Cod liver oil.....	0.5	10.0		+	
" " "	1.0	5.0	+	+	
" " "	1.0	4.0	(±)		Test within a few min. of making preparation. See Rows 11 and 12 below.
" " "	1.0	4.0		+	
" " "	1.0	3.6	+	+	
Mineral "	1.0	3.6	−		
Cod liver "	1.0	3.6	+		
Olive oil.....	1.0	2.8	+	+	
Cod liver oil.....	1.0	2.8	±	±	
" " "	1.0	2.5		±	
" " "	1.0	2.0	±		Test within a few min. of making preparation.
" " "	1.0	2.0	+	+	Same preparation 90 min. later.
" " "	1.0	2.0	±	±	
" " "	1.0	1.2	−	±	
Mineral "	1.0	1.2	−	−	
Cod liver "	1.0	1.2	−	−	
Olive oil.....	1.0	1.2		−	
" "	1.0	0.6		−	

been tested for stabilization reaction titers. The two sera of each pair were tested at any given concentration with the same red cell suspension and with experimental conditions as nearly identical as possible. The tests with one pair are given in Table I.

The stabilization titers are evidently about the same for the two

sera as are also the lysin titers determined by Landsteiner and van der Scheer.[9] The agglutination titers on the other hand are quite different. The results with this and the other pair of sera are summarized in Table II.

It will be appreciated that the criteria for the stabilization reaction cannot be applied with quantitative precision, and that the ratios given are approximate only. Nevertheless we are convinced of the correspondence of the stabilization and lysin ratios within the rather large error of the method and of the failure of the stabilization and agglutination ratios to correspond even approximately within the error of the method. Corroborative evidence is furnished by the fact

TABLE II.

Agglutinin titer,	Serum	40:	Serum	32 = 1:6–8
Lysin titer,	"	40:	"	32 = 1:1
Stabilization titer,	"	40:	"	32 = 1:1
Agglutinin "	"	15:	"	30 = 1:8
Lysin titer,	"	15:	"	30 = 1:2
Stabilization titer,	"	15:	"	30 = 1:1–3

Nos. 40 and 15 extract immune sera; Nos. 32 and 30 whole erythrocyte immune sera.

Sensitivity, stabilization reaction : lysis = 1:3–8 (*i.e.* lysis was obtained by Landsteiner and van der Scheer with serum dilutions 3 to 8 times those required to give the stabilization reaction).

that there was agglutination of the red cells suspended in the whole erythrocyte antisera even when the stabilization reaction was negative. Agglutination was regularly much less in the corresponding extract immune sera suspensions, and sometimes gross agglutination or even occasionally microscopic agglutination could not be detected when the stabilization reaction was positive.

The altered behavior of sensitized horse erythrocytes thus depends predominantly upon the hemolytic sensitizer rather than upon the the agglutinin. It is difficult to escape the conclusion (already reached by Landsteiner and his coworkers on other grounds[9]) that the hemolytic sensitizer reacts predominantly with lipoid components of the red cell surface. It would seem also that the agglutinin reacts predominantly with the protein moiety of the erythrocyte surface.

Specifically hemolyzed stromata were prepared by making a 2 per cent suspension of human cells in 4 per cent anti human-erythrocyte rabbit serum and adding fresh human whole blood. The resulting ghosts behaved like strongly sensitized unhemolyzed cells. Even single ghosts bent back the interface. Stromata resulting from 1 per cent horse cell suspensions in 6 per cent anti-horse sera 16 and 31 mixed with equal volumes of 10 per cent fresh guinea pig serum likewise showed strong stabilization reactions.[11] In contrast to the altered interfacial behavior of the strongly sensitized stromata was the normal behavior of stromata obtained by hemolysis with distilled water or by cold during an overnight stay in the ice box. The unsensitized ghosts went over into the oils as easily as ordinary red cells or more so.

Behavior of Leucocytes and Platelets.

During the observation of preparations with the Bausch and Lomb dark-field condenser with substage lamp the appearance and behavior of the human leucocytes encountered was as follows:

1. Cells rounded in outline in which granules could often be observed in movement. These cells entered the boundary surface with little difficulty and were spread along the interface with complete disintegration. Some of the component granules streamed along the interface in both directions away from the site of disintegration, where the liquid-liquid interfacial tension was obviously lowered, and others passed over into the oil. Less frequently a rapidly advancing interface swept over such a leucocyte, leaving it somewhat spread and disintegrated in the oil.

2. Cells irregular in outline in which movement of granules was not seen. Such cells were often pushed for many minutes before the advancing interface. If the white cell was stopped by adhesion to the glass or was overtaken by a too rapidly advancing interface it bent the interface back into a "peninsula" before being dragged into the oil. Often the leucocyte in the distal end of such a peninsula was

[11] Apparently negative reactions were obtained, however, with some ghosts from partially hemolyzed horse cell-antisera mixtures in which the unhemolyzed cells appeared to give positive reactions.

left in the oil surrounded by a film of aqueous phase. A peninsula about to break off thus is shown in Fig. 4; the leucocyte has been partially dragged out of the vacuole into the oil.

Leucocytes in preparations examined over the Zeiss condenser with carbon arc and heat filter showed almost exclusively the behavior described under (1) above. An illustrative protocol is given.

Small drop of Ringer-Tyrode solution put on slide; tiny drop of blood fresh from finger added. Oil drop put on other half of slide and cover-slip superposed. Preparation observed with Zeiss condenser (bright field, which shows better than the dark field the distinctions between the leucocyte cell types), arc, and heat filter. Red cells seen to snap across into oil in usual way. Many white cells studied, mostly granular leucocytes; these were in healthy condition. When hit by interface they entered it, formed lenses, and disintegrated, spreading up and down interface; when this happened the interface would run forward locally as disintegrated protoplasm spread. Some white cells formed lenses and passed directly into oil, disintegrating as they were enveloped by the oil. An eosinophil was seen to enter the interface, form a lens, and slide along the interface about one oil immersion field; it then spread in the interface and some of the large eosinophil granules drifted over into the oil. The preparation was now transferred to the microscope with substage lamp. At first a few normal looking polymorphonuclear leucocytes were seen to act in the manner described in the first part of the experiment but gradually they came to look more pronouncedly granular and less liquid, more hard and irregular in outline. As this process progressed the cells at first bent back the interface very slightly and were disintegrated as they passed into the oil; then bent the interface back more and dragged through into the oil, and finally acted in the typical manner described under (2) above.

The altered behavior of leucocytes viewed with the substage lamp appears to be due to the heating of the film by this type of illumination. A thermal junction (made by soldering together constantan No. 40 and copper No. 32 wire and flattening with a hammer) was introduced into films like those described; the other junction was put in ice water, and the electromotive force determined with a potentiometer. The temperatures reached in three different experiments over the condenser with substage lamp were: 53.8°C. in 29 minutes, 57.8° in 44 minutes, 58.9° in 33 minutes. The first preparation only reached 2° or 3° above room temperature when illumined by the carbon arc with water trough heat filter.

Behavior (1) may be regarded as normal for white blood cells under

the experimental conditions described and behavior (2) as that of heat-fixed white cells. The surface tension relations could not with certainty be determined for the normal leucocytes because of their inability to resist spreading. Our belief is, however, that the conditions of equilibrium in the interface are fulfilled; *i.e.*, $T_{so} < T_{sw} + T_{ow}$ and $T_{sw} < T_{so} + T_{ow}$. After heat fixation the leucocytes are extraordinarily resistant to any wetting whatsoever by the oil; they are apparently not in equilibrium in the interface, and $T_{so} > T_{sw} + T_{ow}$.[12] Human leucocytes thus appear to be more labile than erythrocytes both in their resistance to the interfacial stresses and in their resistance to surface alteration with elevation of temperature. We have not adequately studied leucocytes of other species. Kite[13] from micro dissection studies has also concluded that leucocytes are naked protoplasm unprotected by any morphologically differentiated surface layer.

Rabbit blood platelets, like the human leucocytes, became increasingly refractory to wetting by the oils (mineral and cod liver oils and melted vaseline) during exposure to the heat of the substage lamp.

Fresh platelets, prepared by drawing 4 cc. of rabbit blood directly into 1 cc. of 1.3 per cent sodium oxalate solution, and mixing and separating the platelets by centrifugation, were made available daily through the kindness of Dr. C. M. Van Allen. Diluted with NaCl and studied with the heat filter or during the first minutes of observation with substage lamp, the platelets floated through the interface as though it offered little if any obstruction. The platelets did not form lenses or spread in the interface. After a few minutes over the substage lamp the platelets were much more stable in the interface. They slid along the interface for longer distances, left it only at the expense of mechanical work, and bent back the interface in so doing.

Minute, brightly shining granules were seen regularly in the preparations of blood and serum. These adhered readily to the glass and

[12] This result is remarkable in that the alteration of the leucocytes with heating is in just the opposite sense to that which would be anticipated from denaturation of the proteins of the protoplasm (Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1925–26, ix, 169). It suggests either a redistribution of substances in the surface layers of the cell or chemical modification of non-polar surface groups to form polar groups.

[13] Kite, G. L., *J. Infect. Dis.*, 1914, xv, 319.

when overtaken by the interface pulled it into sharp pointed peninsulas. One of these is shown in the middle of the interfacial line in Fig. 1, *a* and *b*, and two appear in the upper left hand corner of Fig. 4. The behavior of these granules in the interfaces shows their surfaces at least not to be fat-like. They seem to be among those comprised under the term hemokoniæ or blood dust.[14] However, they are clearly different from the spherules seen in the turbid serum of animals bled without having been previously fasted (alimentary lipemia). The existence of these two types of refractile particles in sera doubtless accounts for some of the confusion in the literature regarding hemokoniæ.

Behavior of Some Other Particles.

Pure crystals of oxyhemoglobin given us by Dr. M. Heidelberger were suspended in distilled water and studied against the oils (triolein, tricaprylin, mineral, cod liver, sperm, and cedar oils). They were stable in the interfaces, slid along them, and bent back the boundary line when dragged through into the oils.

Potato-starch (Kahlbaum's) and corn-starch grains suspended in distilled water were swept before the advancing oils (triolein, tricaprylin, mineral, and cod liver oils). They were not stable in the interfaces and were in no instance observed to cross into the oil except when a vacuole of water containing starch grains was left behind at the end of a peninsula.

Shreds of ground up filter paper (Baker and Adamson, ash-free) in distilled water were pushed before the interfaces and bent the interface into peninsulas if dragged through into the oils (triolein, tricaprylin, tributyrin, mineral, and cod liver oils).

The interfacial tension relations of a number of cells and cell products in aqueous suspension in contact with the specified organic phases (references 1 and 2 and this paper) fall into the following categories.

[14] Krehl, L., and Marchand, F., Handbuch der allgemeinen Pathologie, Leipsic, 1912, ii, pt. 1, 215. Hewlett, A. W., Functional pathology of internal diseases, New York and London, 1917, 243. Stengel, A., and Fox, H., A textbook of pathology, Philadelphia and London, 7th edition, 1921, 436.

1. These particles are wet by water but not by oil. They are not in equilibrium in the organic-aqueous phase interface. $T_{so} > T_{sw} + T_{ow}$:

Heat-fixed leucocytes; starch grains; certain granules in serum; cellulose (filter paper) (?).

2. These particles are in equilibrium in the organic-aqueous phase interface. $T_{so} < T_{sw} + T_{ow}$ and $T_{sw} < T_{so} + T_{ow}$:

(*a*) Escape into the aqueous phase is relatively easy. The inequality $T_{so} < T_{sw} + T_{ow}$ is small. $T_{so} > T_{sw}$:

Ordinary bacteria;[2] mammalian erythrocytes and stromata after strong specific sensitization; oxyhemoglobin crystals (?); cellulose (?).

(*b*) Relative ease of escape into organic and aqueous phase not certain:

Fresh human leucocytes; heated rabbit platelets; oxyhemoglobin crystals (?).

(*c*) Escape into oil is relatively easy. The inequality $T_{sw} < T_{so} + T_{ow}$ is small. $T_{sw} > T_{so}$:

Acid-fast bacteria against most oils; unsensitized mammalian erythrocytes and stromata; some preparations of fresh oxalated rabbit platelets.

3. These particles are wet by the organic phase but not by the aqueous phase. They are not in equilibrium in the interface. $T_{sw} > T_{so} + T_{ow}$:

Acid-fast bacteria with oleic acid and tricaprylin; some preparations of unheated oxalated rabbit platelets (?).

DISCUSSION.

Mammalian erythrocytes possess a surface pellicle[15] which often becomes wrinkled in our films. Such cells may be seen to be pulled out by the interfacial stresses into lens-shaped bodies with smooth surfaces. The red cells are not spread with disintegration by the interfacial stresses as are normal human leucocytes. As to whether all or only a part of the erythrocyte pellicle is concerned with the cell permeability the present work affords no data.

The surface of erythrocytes is capable of being drawn out into delicate processes. These have been described previously by Noguchi[16] with cells in process of hemolysis by snake venom, by Kite[13] and by Mathews[17] with untreated cells, and by Rous and Robertson[18] with human red cells more especially from patients with pernicious anemia.[19]

[15] Ponder, E., *Proc. Roy. Soc. London, Series B*, 1924–25, xcvii, 138.

[16] Noguchi, H., Snake venoms, *Carnegie Institution of Washington, Pub. No. 111*, 1909, 170.

[17] Mathews, A. P., Physiological chemistry, New York, 2nd edition, 1916, 465.

[18] Rous, P., and Robertson, O. H., *J. Exp. Med.*, 1917, xxv, 651.

[19] Rous, P., personal communication; also *Physiol. Rev.*, 1923, iii, 75.

Such processes may also be seen extending from the distorted stromata obtained by prolonged centrifugation at high speed of laked blood. The formation of surface processes in our preparations is greatly accentuated by treatment with specific anti-erythrocyte serum.

In an interesting study on the electric capacity of blood, Fricke[20] has lately drawn the conclusion, with the aid of certain additional assumptions, that the surface membrane of mammalian erythrocytes is only monomolecular in thickness. This view we cannot harmonize with the facts just mentioned. Moreover, Seifriz[21] has been able to stretch the stromata between micro dissection needles; from an amphibian erythrocyte he can actually release cytoplasm and nucleus and then stretch the elastic pellicle.

Analytical studies have shown that the stromata of erythrocytes contain mineral salts, protein, and lipoid, cholesterol and lecithin constituting the greater part of the lipoid.[22] The presence in the pellicle of lipoid and protein is also indicated by ultramicroscopic observation.[23] This makes it probable although not certain that normal red cells present to the plasma surfaces with both lipoid and protein components.

Evidence indicating the presence of protein in the erythrocyte surface is furnished by cataphoretic and titration data of Coulter's[24] showing an isoelectric point at pH = 4.6. The shape of the titration curves and the position of the isoelectric point[25] suggest that material other than the stroma globulin is also present in the surface. The well known fact that substances which attack lipoids are hemolytic also bespeaks a part for lipoids in the erythrocyte surface.

The work of Bang and Forssman, Landsteiner and his collaborators, and others[9] indicates also the dual nature of the antigens of the red cell surfaces. Alcohol and ether extracts of red cells give rise to and react with antisera with relatively small agglutinin:lysin ratios; the lipoid-insoluble residues show protein reactions and give rise to agglutinins.

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[20] Fricke, H., *J. Gen. Physiol.*, 1925–26, ix, 137.

[21] Seifriz, W., personal communication.

[22] Pascucci, O., *Beitr. chem. Physiol. u. Path.*, 1905, vi, 543, 552. Wells, H. G., Chemical pathology, Philadelphia and London, 4th edition, 1920, 211. Hamburger, H. J., *Ergebn. Physiol.*, 1924, xxiii, pt. 1, 129.

[23] Salén, E., *Biochem. Z.*, 1920, cx, 176.

[24] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.

[25] Mudd, S., *J. Gen. Physiol.*, 1924–25, vii, 389.

Further corroborative evidence is furnished by our observation that normal erythrocytes pass readily but not spontaneously from the interfaces into oil; that specifically sensitized erythrocytes are no longer readily miscible with oil; and that this increase in polarity of the erythrocyte surface on sensitization corresponds fairly well to the hemolysin titer ratios and is relatively independent of agglutinin titer.

The recent contention of Gorter and Grendel[26] that erythrocytes are covered with a layer of lipoids just two molecules thick, aside from its *a priori* improbability, is in direct conflict with several of the above facts. It is clear that the unsensitized erythrocyte surface contains both lipoid and protein. The changes produced in this surface by treatment with immune sera have already been discussed.

SUMMARY.

The interfacial tension method has been applied to the study of the surface composition of mammalian blood cells and to certain other particles.

Unsensitized erythrocytes and stromata possess only a small margin of stability in the interface and pass readily into the oil phase.

Specifically sensitized erythrocytes and stromata possess much greater stability in the interface and pass into the oil only with considerable mechanical aid; characteristic deformations of the erythrocyte surface or the interface or both often result.

With special immune sera prepared by Landsteiner and van der Scheer[9] the quantitative relations are such as to indicate that the increased polarity of the sensitized erythrocyte surface is due to combination of the red cell surface lipoids with hemolytic sensitizer. These results are corroborative of the conclusion of Landsteiner and van der Scheer that erythrocytes contain specific lipoid-soluble antigens.

The tentative conclusion is reached that with these anti horse-erythrocyte sera at least the agglutinins combine predominantly with the protein of the red cell surfaces.

Fresh human leucocytes are spread and disintegrated by the interfacial stresses. After heat injury over the condenser with substage

[26] Gorter, E., and Grendel, F., *J. Exp. Med.*, 1925, xli, 439.

lamp the leucocytes typically do not enter the boundary surface. They are pushed before the advancing interface and, if their further advance is obstructed, bend the interface backward to form peninsulas and vacuoles. This change after heating is in the opposite sense to that to be expected from denaturation of the proteins of the protoplasm.

Fresh oxalated rabbit platelets pass very easily into the oils. After heating over the substage lamp these elements also become less oil-miscible.

The interfacial tension relations of blood cells, bacteria, and several cell products are tabulated.

We would express our gratitude to the investigators who have given us difficultly obtainable materials and valuable advice; and our appreciation also of the skill of Mr. H. Rosenberger and Mr. L. Schmidt in taking the dark-field moving pictures.

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. Cinematograph of normal red blood cell passing through interface. Cod liver oil on left; human blood diluted with 0.9 per cent NaCl on right; bright line, boundary surface between them. Arrow indicates direction in which interface is advancing.

FIG. 2. Sensitized red cells. Cod liver oil on right; horse cells, 0.5 per cent, in 33 per cent anti horse-erythrocyte rabbit serum, on left. Two pear-shaped red cells in interface. Whole field slightly out of focus.

FIG. 3. Clump of sensitized red cells bending back interface. Slightly out of focus.

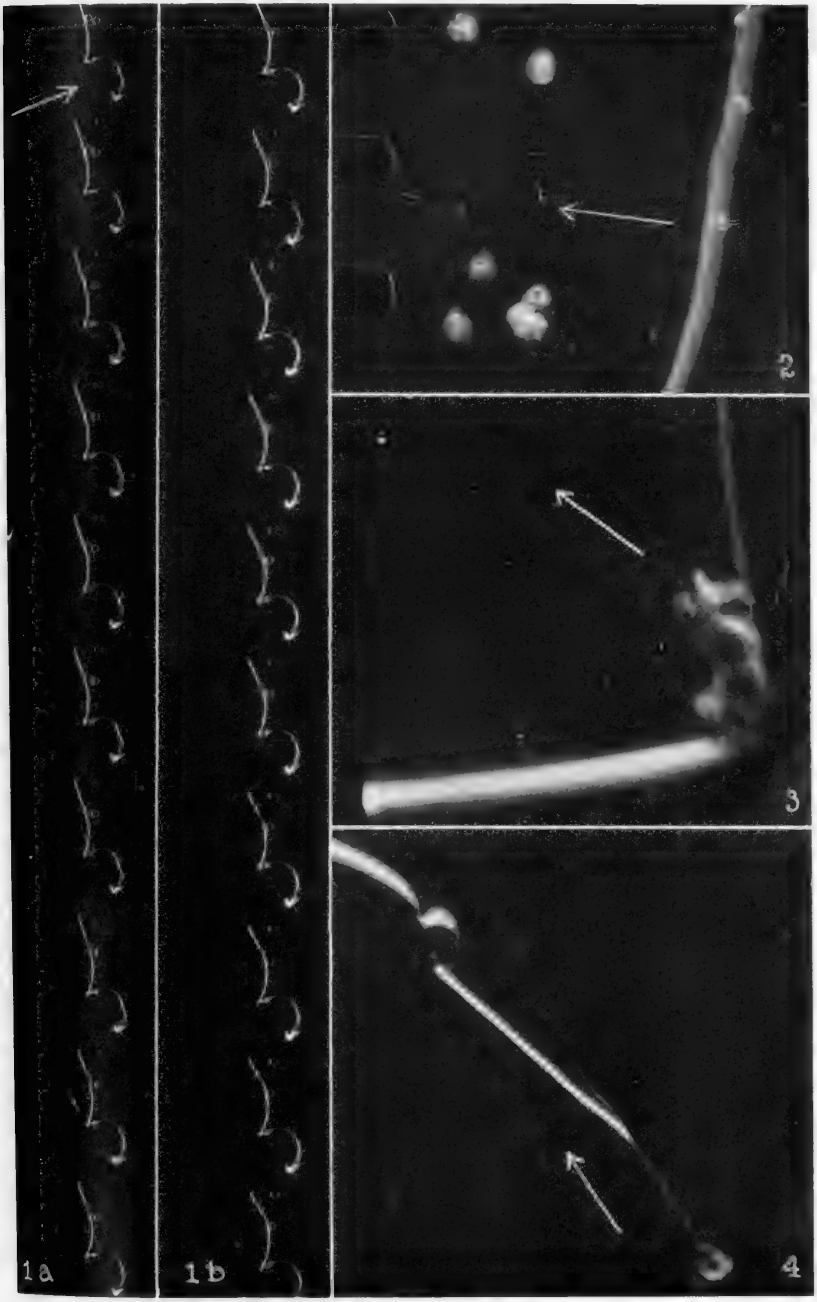
FIG. 4. Heated human leucocyte in a vacuole of dilute plasma about to be left behind in olive oil.

PLATE 3.

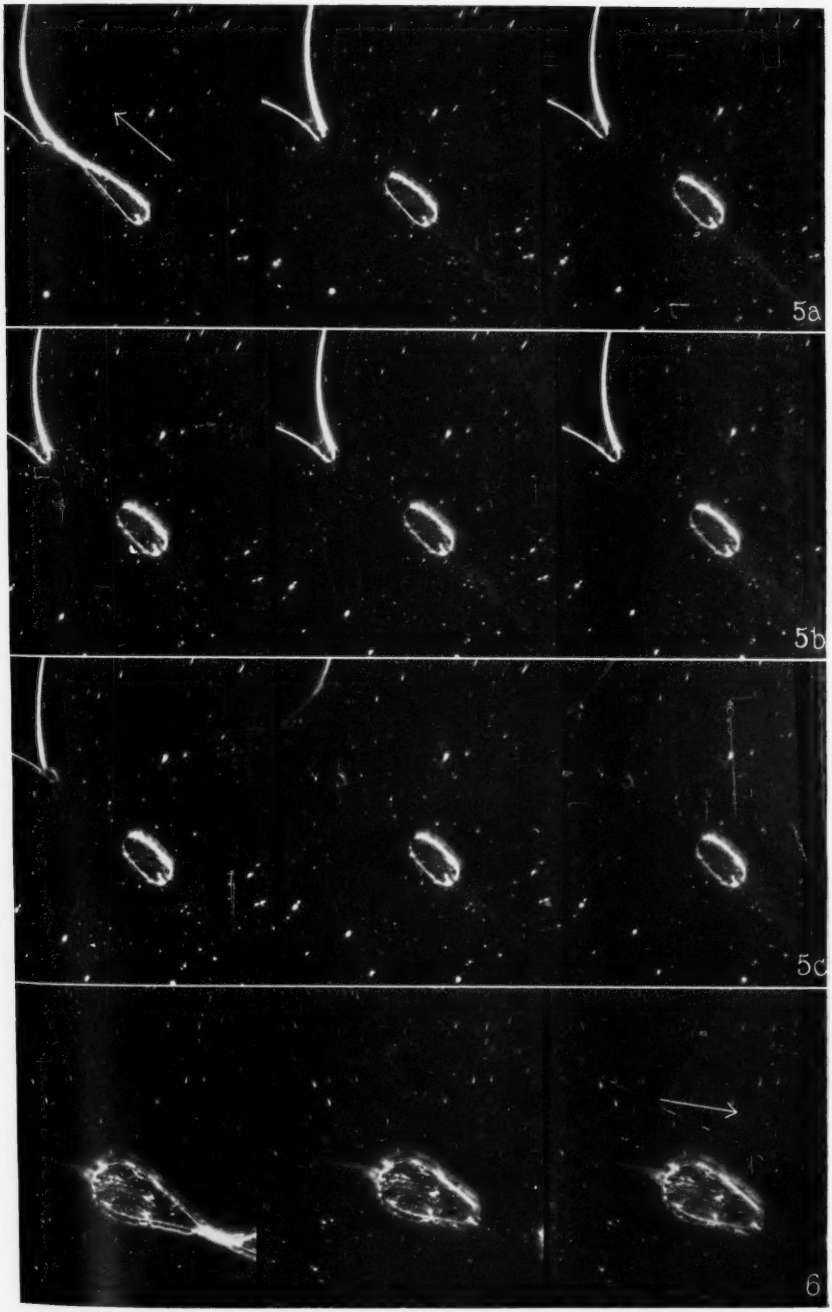
FIG. 5. Cinematograph of interface leaving a vacuole behind and dragging over a small clump of sensitized red cells. Triolein against 1 per cent suspension of horse cells in 0.8 per cent anti horse-erythrocyte serum 30.

FIG. 6. Vacuole and tails. Same materials as in Fig. 5.

The oblong spaces at the sides of Figs. 2, 3, and 4 are perforations in the moving picture film.



(Mudd and Mudd: Surface composition of mammalian blood cells.)



(Mudd and Mudd: Surface composition of mammalian blood cells.)

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